THE MECHANISM OF ACTION OF INSULIN

A SYMPOSIUM

organized by

THE BRITISH INSULIN MANUFACTURERS

Allen & Hanburys Ltd *Boots Pure Drug Co Ltd*
British Drug Houses Ltd *Burroughs Wellcome & Co Ltd*

Consulting Editor

F G YOUNG
MA DSc FRS

Editors for the British Insulin Manufacturers

W A BROOM
DSc FRIC
Boots Pure Drug Co Ltd
Nottingham

F W WOLFF
MD
The Wellcome Foundation
The Post Graduate Medical School London

BLACKWELL
SCIENTIFIC PUBLICATIONS
OXFORD

Published simultaneously in the United States of America by Charles C Thomas Publisher 301 327 East Lawrence A enue Springfield Illinois

Published simultaneously in Canada by the Ryerson Press Queen Street West Toronto 2

PRINTED IN GREAT BRITAIN IN THE CITY OF OXFORD
AT THE ALDEN PRESS
AND BOUND BY THE KEMP HALL BINDERY OXFORD

LIST OF PARTICIPANTS

D W Adamson
The Wellcome Research Laboratories Langley Court Beckenham Kent

Joyce Baird
Department of Therapeutics the Clinical Laboratory the Royal Infirmary Edinburgh 3

O Behrens
Biochemical Research Division Lilly Research Laboratories Indianapolis 6 Indiana

P M Beigelman
Department of Medicine University of Southern California Los Angeles

S P Bessman
Department of Medicine University of Maryland Baltimore Maryland

K. O Black
6 Harley House Upper Harley Street London N W 1

R R. Bomford
59 Philpot Street London E 1

W A Broom
Boots Pure Drug Co Ltd Nottingham

W J H Butterfield
Clinical Research Unit Guy's Hospital London S.E 1

R Catanzaro
Istituto Superiore di Sanità Viale Regina Elena 299 Rome

E B Chain
Istituto Superiore di Sanità Viale Regina Elena 299 Rome

Anne Beloff-Chain
Istituto Superiore di Sanità Viale Regina Elena 299 Rome

A M Cooke
123 Woodstock Road Oxford

*C F Cori
Washington University School of Medicine St Louis Missouri

Sir Henry Dale
The Wellcome Trust 52 Queen Anne Street London

P F D'Arcy
Allen & Hanburys Ltd Ware Hertfordshire

Elizabeth Dennes
Postgraduate Medical School Ducane Road London W 12

*F Dickens
Courtauld Institute of Biochemistry Middlesex Hospital Medical School London W 1

Published simultaneously in the United States of America by Charles C Thomas Publisher 301 327 East Lawrence Avenue Springfield Illinois

Published simultaneously in Canada by the Ryerson Press Queen Street West Toronto 2

PRINTED IN GREAT BRITAIN IN THE CITY OF OXFORD
AT THE ALDEN PRESS
AND BOUND BY THE KEMP HALL BINDERY OXFORD

LIST OF PARTICIPANTS

D W ADAMSON
The Wellcome Research Laboratories Langley Court Beckenham Kent

JOYCE BAIRD
Department of Therapeutics the Clinical Laboratory the Royal Infirmary Edinburgh 3

O BEHRENS
Biochemical Research Division Lilly Research Laboratories Indianapolis 6 Indiana

P M BEIGELMAN
Department of Medicine University of Southern California Los Angeles

S P BESSMAN
Department of Medicine University of Maryland Baltimore Maryland

K O BLACK
6 Harley House Upper Harley Street London N W 1

R. R. BOMFORD
59 Philpot Street London E 1

W A BROOM
Boots Pure Drug Co Ltd Nottingham

W J H BUTTERFIELD
Clinical Research Unit Guy's Hospital London S.E 1

R. CATANZARO
Istituto Superiore di Sanità Viale Regina Elena 299 Rome

E. B CHAIN
Istituto Superiore di Sanità Viale Regina Elena 299 Rome

ANNE BELOFF-CHAIN
Istituto Superiore di Sanità Viale Regina Elena 299 Rome

A M COOKE
123 Woodstock Road Oxford

*C F CORI
Washington University School of Medicine St Louis Missouri

SIR HENRY DALE
The Wellcome Trust 52 Queen Anne Street London

P F D'ARCY
Allen & Hanburys Ltd Ware Hertfordshire

ELIZABETH DENNES
Postgraduate Medical School Ducane Road London, W 12

*F DICKENS
Courtauld Institute of Biochemistry Middlesex Hospital Medical School London W 1

SIR CHARLES DODDS
Courtauld Institute of Biochemistry Middlesex Hospital Medical School London W 1

*C DE DUVE
Université de Louvain Laboratoire de Chimie Physiologique 6 Rue des Doyens Louvain

R B FISHER
Department of Biochemistry University of Oxford South Parks Road Oxford

*T RUSSELL FRASER
Postgraduate Medical School Ducane Road London W 12

I KELSEY-FRY
Guy s Hospital Medical School St Thomas s Street London S E 1

H J GALBRAITH
Department of Medicine St Thomas s Hospital Medical School London S E 1

JEAN GINSBURG
Department of Medicine St Thomas s Hospital Medical School London S E 1

H K GOADBY
St Thomas s Hospital London S E 1

C HARDWICK
Guy s Hospital St Thomas s Street London S E 1

F HARTLEY
British Drug Houses Ltd Graham Street City Road London N 1

G I HOBDAY
Boots Pure Drug Co Ltd Nottingham

J J HOET
Hopital St Pierre 69 Rue de Bruxelles Louvain

J P HOET
Hopital St Pierre 69 Rue de Bruxelles Louvain

L H HOWELLS
71 Cathedral Road Cardiff

W P U JACKSON
University of Cape Town, Medical School Groote Schuur Hospital Observatory Cape Town S Africa

MARJORIE K JEACOCK
Department of Biochemistry University of Cambridge Tennis Court Road Cambridge

A KORNER
: *Department of Biochemistry University of Cambridge Tennis Court Road Cambridge*

M E KRAHL
: *951 East 58th Street Chicago 37 Illinois*

G H LATHE
: *Department of Chemical Pathology the School of Medicine Leeds 2*

R LEVINE
: *Department of Medicine Michael Reese Hospital 29th Street and Ellis Avenue Chicago 16 Illinois*

J LISTER
: *90A Harley Street London W 1*

C N H LONG
: *Department of Physiology Yale University School of Medicine 333 Cedar Street New Haven 11 Connecticut*

R MAHLER
: *Department of Pharmacology and Therapeutics Queen s College Dundee*

J MALINS
: *58 Calthorpe Road Edgbaston Birmingham 15*

K L MANCHESTER
: *Department of Biochemistry University of Cambridge Tennis Court Road Cambridge*

I MASI
: *Istituto Superiore di Sanità Viale Regina Elena 299 Rome*

J M MCFIE
: *4 The Ropewalk Nottingham*

P J MOLONEY
: *Department of Chemistry School of Hygiene University of Toronto Toronto*

H MORGAN
: *Department of Physiology Vanderbilt University School of Medicine Nashville Tennessee*

I MURRAY
: *48 Berkley Street Glasgow 3*

J D N NABARRO
: *121 Harley Street London W 1*

D S H W NICOL
: *Government Laboratory Services c/o Connaught Hospital Freetown Sierra Leone British West Africa*

W OAKLEY
149 Harley Street London W 1

C R PARK
Department of Physiology Vanderbilt University School of Medicine Nashville Tennessee

V PITROW
British Drug Houses Ltd Graham Street City Road London N 1

F POCCHIARI
Istituto Superiore di Sanità Viale Regina Elena 299 Rome

J D PROCTOR
16 Park Terrace Nottingham

R REID
M R C Clinical Chemotherapeutic Research Unit Western Infirmary Glasgow W 1

A E RENOLD
Baker Clinic Research Laboratory New England Deaconess Hospital 185 Pilgrim Road Boston 15 Massachusetts

F A ROBINSON
Allen & Hanburys Ltd Ware Hertfordshire

E J ROSS
Medical Unit University College Hospital Medical School University Street London W C 1

P SCHAMBYE
Novo Terapeutisk Laboratorium A/S 115 Fuglebakkevej Copenhagen N Denmark

J SHIPP
Department of Biochemistry University of Oxford South Parks Road Oxford

G A SMART
University of Durham Medical School King s College Newcastle-upon Tyne

G HOWARD SMITH
Department of Biochemistry University of Cambridge Tennis Court Road Cambridge

G A STEWART
Burroughs Wellcome & Co Biological Control Laboratories Acacia Hall Dartford

J M STOWERS
Department of Medicine Queen s College Dundee

K W TAYLOR
Department of Biochemistry University of Cambridge Tennis Court Road Cambridge

J TERPSTRA
Academisch Ziekenhuis Leiden Inwendige Geneeskunde Afdeling Stofwisselingsziekten en Endocrinologie Leiden

R E TUNBRIDGE
Department of Medicine the General Infirmary Leeds 1

J VALLANCE OWEN
University of Durham Medical School King s College Newcastle upon Tyne

JOAN WALKER
40 Springfield Road Leicester

G M WILSON
Department of Pharmacology and Therapeutics the University Sheffield 10

†F W WOLFF
The Department of Medicine Johns Hopkins Hospital Baltimore Md U S.A

P H WRIGHT
Department of Chemical Pathology Guy s Hospital Medical School London S E 1

B A YOUNG
St Alfege s Hospital London S.E 10

D A B YOUNG
Department of Biochemistry University of Oxford South Parks Road Oxford

*F G YOUNG
Department of Biochemistry University of Cambridge Tennis Court Road Cambridge

P ZACHARIAH
Department of Biochemistry University of Oxford South Parks Road Oxford

Together with certain members of the scientific staff of the British Insulin Manufacturers

* Session Chairman.
† Symposium Secretary

CONTENTS

PART IV

Insulin and the Liver

Chairman Professor C F Cori

PART V

Insulin and Protein Synthesis

Chairman Professor F Dickens

PART VI

Bio-Assay of Insulin

Chairman Professor T Russell Fraser

PART VII

Insulin Antagonists

Chairman Professor T Russell Fraser

PART VIII

Mechanism of Action of Insulin and other Hypoglycaemic Substances

Chairman Professor C de Duve

PART IX

Clinical Aspects of Diabetes and Pre-Diabetes

Chairman Professor F G Young

INTRODUCTION

F G YOUNG

It is at once a salutary fact and a challenge to research that nearly 40 years after the discovery of insulin much disagreement still exists about the mechanism of action of this important hormone The annual volume of publications on this subject is enormous and is still rapidly growing nevertheless ideas about the way in which insulin acts appear to steer an unsteady course between the Charybdis of sugar transport and the Scylla of direct action on identifiable enzyme systems with occasional sidelong glances at other possibilities Perhaps in the end Charybdis will engulf Scylla and we shall understand the action of insulin in terms of a promotion of transport of glucose into a cell by virtue of an influence of insulin on certain enzymes or co-enzymes which are themselves concerned with the transport of sugar across permeability barriers of the cell Already a few rays of light shine in this hopeful direction and the account of the contributions and discussions recorded in this volume shows that there need be no deep dissatisfaction with the progress made in recent years

Although meetings for the discussion of current research and the subsequent publication of a record of the contributions and cussion are becoming not uncommon these days the meeting which this volume is the published record differed from many otr in two respects The subject of discussion was in theory a narro one although in practice the discussions were wide while th taking part included both chemists and clinicians with a c interest Insulin was the focus of all providing the cohesive which held the meeting together in vigorous discussion for successive days

The absence of Professor Loubatières who was kept away last moment by family illness was most disappointing but present were deeply indebted to Rachmael Levine for filling at the last moment and giving a masterly survey of the Professor Loubatières and others on the action of anti-sulphonamides

Although a full record of the discussions was made the of the meeting agreed at the beginning that anybody was excise from the record remarks which on due reflection ferred not to be recorded in print With this in mi

urged to be informal and frank in their discussions and not to hesitate to ask simple questions as well as those that might display the erudition of the questioner The simple questions can sometimes be the most penetrating How far this exhortation was effective can be seen from the following pages but it is refreshing to note that very little of the recorded discussions was in fact excised by the contributors

The expenses of the meeting including much of the travel costs of the members were provided by the British Insulin Manufacturers This organization was formed as the result of an agreement between the four manufacturers of insulin in Great Britain (Allen & Hanburys Ltd Boots Pure Drug Co Ltd British Drug Houses Ltd and Burroughs Wellcome & Co Ltd) to provide for exchange of information and the fostering of research on insulin The conference was the first held under the auspices of the British Insulin Manufacturers of which an account is published The editing of the record was undertaken jointly by Mr W A Broom of Boots Pure Drug Co Ltd and Dr F W Wolff of Burroughs Wellcome Ltd Dr Wolff also undertook most successfully the detailed administrative duties involved in the organization of the conference On behalf of the British Insulin Manufacturers the Chairman of their Technical Committee Dr G I Hobday of Boots Pure Drug Co Ltd opened the meeting which was held in the Wellcome Building London on September 9th and 10th 1958

All those present hoped that the first meeting of this sort will not prove also to be the last and the published record may reveal some of the reasons for this hope to those concerned in some way or another with the substance that has saved more lives and made an almost normal life possible for more people than any other hormone As was said at the meeting it is indeed fortunate that ability to use insulin therapeutically in no way depends on a detailed knowledge of its mechanism of action But nobody can properly doubt that growth in the understanding of insulin and its ways will inevitably bring with it a wider and more effective therapeutic application The more the laboratory worker and the clinician understand each other s tasks the more quickly the results of research can become fruitful in the treatment of disease This book is published as a contribution to this end

May 2nd 1959 F G YOUNG

PART I

CHEMICAL NATURE OF INSULIN

Chairman PROFESSOR F G YOUNG

THE ACTIVITY OF MODIFIED FORMS OF INSULIN

D S H W Nicol*

Department of Biochemistry University of Cambridge

The purpose of testing the activity of chemical structures related to insulin is to discover the essential site of activity in the molecule as this may help towards revealing the mechanism of its action Another aspect of investigations of this nature which will not be dealt with here is the important one of discovering modifications which are more valuable and convenient in the treatment of diabetes

Although retrospective in time Sanger s formula of insulin[37] will help us to describe briefly previous results in the main field of re-

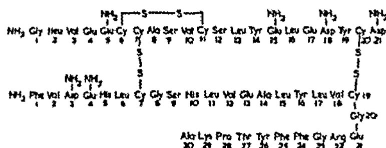


FIG 1 The structure of ox insulin

lating structure to activity (Fig 1) Various reviews are ... on this subject Among them are those of Anfinsen and Redfi... ld[?] Porter[33] and Sanger[39] A prepared table (see Table I) ... of the distinguished work of the past 35 years in the ... modification of insulin would lead to the conclusions that the ...tial groups or linkages were the free carboxyl group the ... hydroxyl groups and the disulphide bonds and that ... essential bonds were the amino groups the guanidino ... amide groups and the aliphatic hydroxyl groups

* Beit Memorial Fellow for Medical Research J R Wolf Medical Fell... College Cambridge

When soon after its discovery insulin was shown to be a protein modification of the molecule by proteolytic enzymes was extensively investigated The use of proteolytic enzymes was important because of their specific nature and the gentle conditions under which they acted

The discoverers of insulin Banting and Best[4] had shown that trypsin destroyed insulin In fact the diminution or absence of proteolytic enzymes from the pancreas was one of the factors which they made use of in their isolation of insulin Other workers confirmed this and the inactivating nature of pepsin and papain [10,41,4,47] Epstein and co-workers did not believe the action of trypsin was proteolytic and claimed to have reactivated insulin which had been subjected to trypsin digestion for 42 hours [11] Their work could not be confirmed however [41] probably because their criterion of insulin action – convulsions in a rabbit – was too diffuse

The tryptic and peptic hydrolyses of insulin were further studied in detail by Charles *et al* [9,10] at the Connaught Laboratories in Toronto and by Freudenberg and his co-workers in Germany [16] Attempts were made to correlate the destruction of physiological activity with the loss of protein The former was found to exceed the latter greatly

More recently Butler *et al* [5,6,7] showed that crystalline chymotrypsin trypsin and pepsin destroyed the activity of insulin. Van Abeele and Campbell[1] found that under mild conditions trypsin did not destroy the activity of insulin and Harris and Li[20] confirmed this showing that only alanine was released

Lens [8] showed the release of alanine from insulin by carboxypeptidase and with this he found that most of the activity had disappeared Harris and Li[20] probably using a more purified sample of enzyme found that activity was retained in the molecule after alanine was removed but that further action resulted in the removal of asparagine and a diminution in the biological activity of insulin

It was at this point that the formula of insulin was finally and completely elucidated by Sanger and his team at Cambridge [37] This opened the way to a more exact assessment of results in this type of investigation Since then Smith *et al* [43] have shown that leucine aminopeptidase removes a considerable number of amino acids from the N-terminal end of insulin without complete impairment of its activity

Our own investigations were directed towards the preparation of

insulin peptides of known structure which could be tested by the rabbit blood sugar method and by the glucose uptake of isolated rat hemidiaphragms Fisher's *t* test was always applied to the latter

The action of carboxypeptidase on insulin was first investigated The enzyme was first incubated with DFP (Di-isopropylfluorophosphoridate) which inactivates any contaminating trypsin and chymotrypsin present Insulin was then added and the enzyme-substrate mixture incubated at pH 7 8 Conditions were obtained in which most of the C-terminal alanine was removed but very little of the asparagine When this was done the activity was unaffected (Fig 2) When however a greater quantity of asparagine was removed there was diminution in biological activity (Fig 3) The work of Harris and Li was thus confirmed.

Sanger and Thompson[40] had shown that the action of carboxypeptidase was more effective with acetyl-insulin as substrate than with insulin Acetyl-insulin (when only the amino groups are acetylated) is active (see Table I) The peptide resulting from the removal of alanine and asparagine by the action of carboxypeptidase showed considerable loss of activity (Fig 4)

The action of trypsin on insulin is at two sites on the phenylalanyl chain (Fig 1) These are beyond the arginyl residue[2 23] and beyond the lysyl residue[29 30] Thus the three products of hydrolysis are a large peptide (DHA insulin we have called it) a heptapeptide Gly Phe Phe Tyr Thr Pro Lys and the terminal alanine molecule Different conditions were used to increase the extent of the hydrolysis The most successful was when the substrate used was insulin which had been treated with versene to remove most of its content of zinc It will be recalled that Smith *et al*[43] first used this low content zinc insulin in their leucine-aminopeptidase experiments After incubation for about 24 hours the tryptic hydrolysate was then brought to pH 5 2 The supernatant of the tryptic hydrolysate contained the heptapeptide and alanine (Fig 5) and these were separated by ionophoresis and identified by paper chromatography after acid hydrolysis The precipitate contained both DHA insulin and unchanged insulin (Fig 6) Separation of both was achieved by an Andersen type column[2] The integrity and composition of DHA insulin was determined by various methods including an analysis by the Moore-Stein method carried out by Mr M Rees and Miss B Worboys of this department and by the DNP N-terminal analysis method[38]

TABLE I

THE ACTIVITY OF SOME CHEMICALLY MODIFIED FORMS OF INSULIN

Authors	*Procedure*	*Groups affected*	*Activity*
Freudenberg *et al* (1931)[16,17]	Acid hydrolysis	Peptide bonds	Inactive
Phillips (1952)[34]	Acid hydrolysis (Conc HCl)	Peptide bonds	Inactive
Mills (1954)[31]	Acid hydrolysis (Conc HCl)	Peptide bonds	Inactive
du Vigneaud *et al* (1931)[47]	Reduction	S-S-bridges	Inactive
Freudenberg *et al* (1935)[19]	Reduction	S-S-bridges	Inactive
Stern and White (1937)[45]	Reduction	S-S-bridges	Inactive
Freudenberg *et al* (1932)[8]	Oxidation	S-S-bridges	Inactive
Freudenberg *et al* (1935)[19]	Oxidation	S-S bridges	Inactive
Fraenkel-Conrat J and H (1950)[4]	Reduction	S-S bridges	Inactive
Harington and Neuberger (1936)[1]	Iodination	Phenolic hydroxyl groups	Inactive
Stern and White (1938)[46]	Acetylation	Phenolic hydroxyl groups	Inactive
Fraenkel Conrat J and H (1950)[4]	Iodine pH 7 6 ~ hr	Phenolic hydroxyl groups	Inactive
Fraenkel-Conrat J and H (1950)[14]	Phenylisocyanate m chlorophenylisocyanate	Phenolic and imidazole groups—extensive	Inactive
Fraenkel-Conrat J and H (1950)[14]	Diazobenzenesulphonic acid	A few phenolic and imidazole groups	Partially active
Jensen *et al* (1928)[21]	Acetylation	Amino and others	Inactive
Freudenberg *et al* (1928 1932)[15, 8]	Acetylation	Amino hydroxyl imino	Partial reversible inactivation
Hopkins and Wormall (1934)[23]	Phenylisocyanate p-bromophenyl isocyanate	Free amino groups	Inactive
Jensen and Evans (1934)[25]	Phenylisocyanate	Amino and hydroxyl groups	Inactive
Stern and White (1938)[46]	Acetylation	Free amino groups	Active
Fraenkel-Conrat J and H (1950)[14]	Acetic anhydride	Amino groups	Active
Fraenkel-Conrat J and H (1950)[14]	Ketene	Amino groups	Active
Fraenkel-Conrat J and H (1950)[14]	M nitrite pH 4 1 30 min	Amino groups	Active
Li (1956)[30]	2 4-dinitrobenzene sulphonate	Amino groups of Lysine	Active
Reitz *et al* (1946)	Sulphation (Conc H SO at —18°)	Aliphatic hydroxyl groups of serine and threonine	Active
Fraenkel-Conrat J and H (1950)[4]	Phosphorylation	Aliphatic hydroxyl groups	Partially active
Freudenberg *et al* (1932)[18]	Formaldehyde	Reduction	Inactive
Scott (19 5)	Formaldehyde	Reduction	Inactive
Fraenkel-Conrat and Olcott (1948)[13]	Formaldehyde at pH 11 12	Amido, Guanidino } groups	Active
Carr *et al* (1929)[6]	Esterification	Free carboxyl groups	Inactive
Freudenberg *et al* (1931)[16]	Esterification	Free carboxyl groups	Inactive
Glendenning *et al* (1947)	Esterification	Free carboxyl groups	Inactive
Mommaerts and Neurath (1950)[35]	Esterification	Free carboxyl groups	Inactive
Kaplan *et al* (1944 1950[28])	Ultra violet radiation	Denaturation	Inactive
Phillips (1952)[34]	Ether trichloracetic acid 5M urea, di isopropyl fluorophosphonate	Physico chemical combination	Active

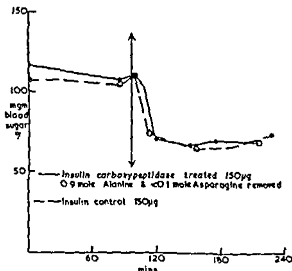


Fig. 2. The peptide resulting fr m the act on of carboxypepod se on insulin (I) — rabb t blood-sugar method.
Time of intravenous injection

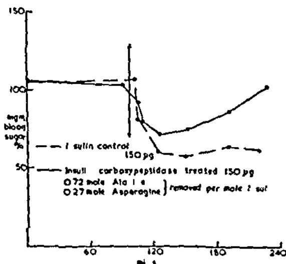


Fig. 3 Th peptide resulting fr m the further action of carboxypeptidase on insulin (II) — r bb t blood-sugar method.
Time of intravenous injection.

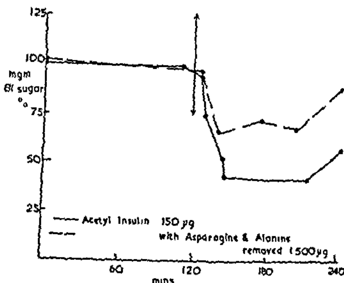


FIG 4 The peptide resulting from the act on of carboxypeptidase on acetyl insul n — rabb t blood-sugar method
Time of intravenou in ection

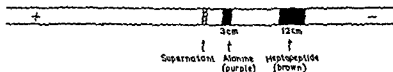
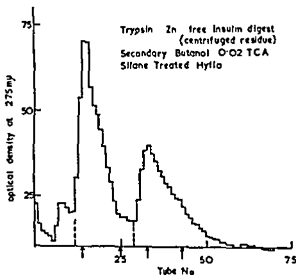


FIG 5 The supernatant fluid from the trypti d gest of insulin at pH 6 2

The assay of the first isolated batch of DHA insulin showed little activity [68] But gentler methods of extraction of the column fluid followed by confirmatory analysis[33a] gave samples of DHA insulin *which showed about 15 per cent activity* (see for example Fig 7) The heptapeptide showed no activity (Fig 8) neither did a mixture of DHA insulin heptapeptide and l-alanine give more than the level of DHA insulin alone (Figs 9 and 10) Complete esterification was



Fi 6 Separat on of DHA insul n (first pe k) f om unchanged insulin by reverse phase column hromatogr phy

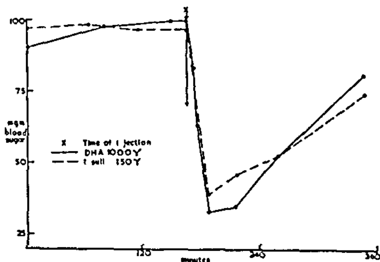


Fig. 7 The ctivity of DHA insulin — rabbit blood-sugar method.
Time of intravenous injection.

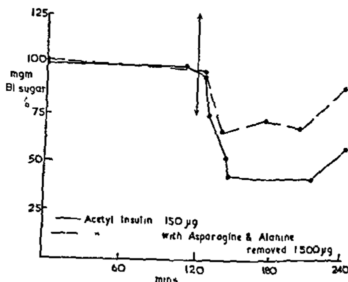


FIG 4 The pept d resulting from the act on of carboxypeptidase on acetyl insulin — rabbit blood sug r meth od
Time of intravenous injection.

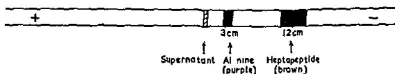


FIG 5 The supernatant flui l from the tryptic digest of insulin at pH 5 2

The assay of the first isolated batch of DHA insulin showed little activity[33] But gentler methods of extraction of the column fluid followed by confirmatory analysis[33a] gave samples of DHA insulin which showed about 15 per cent activity (see for example Fig 7) The heptapeptide showed no activity (Fig 8) neither did a mixture of DHA insulin heptapeptide and l-alanine give more than the level of DHA insulin alone (Figs 9 and 10) Complete esterification was

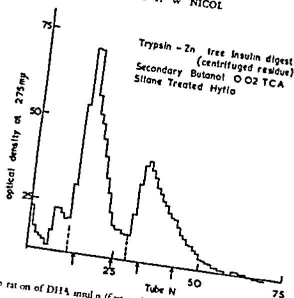


FIG 6 Sep rat on of DHA insul n (f rst peak) f om unchanged insulin by reverse ph se column chrom togr phy

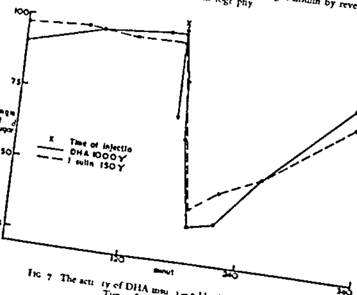


FIG 7 The acti ty of DHA insu i — r bl t blood-sug r method.
Tim of int venou injection.

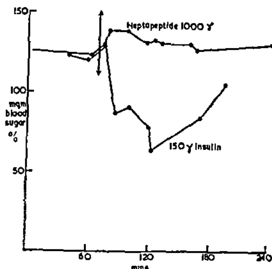


Fig 8 The activity of the heptapeptide Gly Phe Phe Tyr Thr Pro Lys — rabbit blood sugar method
Time of intravenous injection.

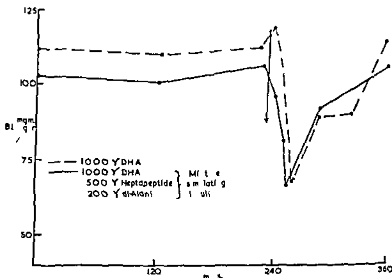


Fig 9 The activity of a mixture of DHA insulin, the heptapeptide and dl alanine compared with that of DHA insulin — rabbit blood sugar method.
Time of intravenous injection

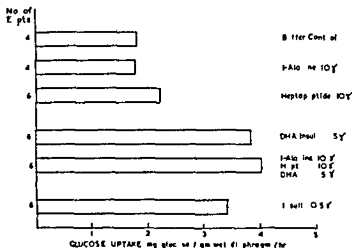


Fig. 10 The activities of the substances resulting from tryptic d gestion of ins lin, separately and together Glucose upt ke of r t diaphr gm method

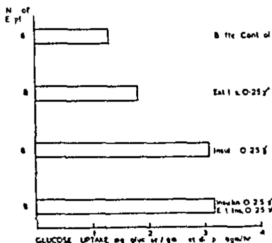


Fig. 11 The act ty of esterified insulin alone and w th insulin. Glucose upt ke of r t d aphr gm method.

carried out by methanolic hydrochloric acid and it was confirmed that activity was lost (Fig 11)

A sample of acetyl insulin donated by Dr L F Smith in which the amino groups but not the hydroxyl groups of tyrosine had been acetylated showed activity Esterified and acetylated insulin have of course been investigated before by other workers (see Table I) and our work has simply confirmed their results using the new assay technique of the glucose uptake of the isolated rat diaphragm

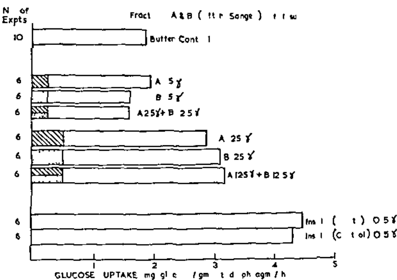


Fig 12 The activity of Fractions A and B (Sanger) on insulin separately and together Glucose uptake of rat diaphragm method

The oxidized glycyl and phenylalanyl chains (Fractions A and B) were prepared and purified by electrophoresis A single experiment showed (Fig 12) an increase in the glucose uptake of the isolated diaphragm but at a level a hundred times less than insulin Together Fractions A and B gave only an additive and not a synergistic effect (Fig 12)

Finally these experiments chiefly those connected with the proteolytic hydrolysis of insulin show the importance of asparagine in the glycyl chain and of the heptapeptide in the phenylalanyl chain

r the maintenance of the full activity of insulin. This may reflect sensitive and essential nature of the region around the second .erchain disulphide linkage of insulin

REFERENCES

VAN ABEELE, F R. & CAMPBELL E. D (1951) *Fed Proc* 10 263
ANDERSEN W. (1954) *Acta chem Scand* 8 359
ANFINSEN C B & REDFIELD R. R (1956) *Adv Prot n Chem* 11 71 Ac demic Press New York.
BANTING F G & BEST C H (1921 22) *J Lab l Md.* 7 251
BUTLER J A V DODDS E. C PHILLIPS D M. P & STEPHEN J M. L. (1948) *Biochem J* 42 116 122
BUTLER J A. V DODDS E. C PHILLIPS D M P & STEPHEN J M L. (194.) *B ochem J* 44 224
BUTLER J A V PHILLIPS D M P STEPHEN J M. L. & CREETH J M (1950) *Biochem J* 46 74
CARR F H CULHANE, K FULLER A T & UNDERHILL, S W F (1929) *B ochem J* 23 1010
CHARLES A F & SCOTT D A. (1930) *T ans oy Soc Can* 24 5 95
DUDLEY H W (1,23) *B ochem J* 17 327
EPSTEIN A A & ROSENTHAL N E. (1924) *Amer J Phys l* 70 225
FISHER A. M. & SCOTT D A (1934) *J biol Chem* 106 289
F AENKEL-CONRAT H & OLCOTT H S (1948) *J b ol Chem* 174 82
FRAENKEL-CONRAT J & FRAENKEL-CONRAT H (1950) *B och m b p y Acta* 5 89
FREUDENBERG K & DIRSCHERL, W (1928) *H ppe-Seyl Z* 175 1
FREUDENBERG K DIRSCHERL W EICHEL, H & WEI E (1931) *H ppe-Seyl Z* 202 159
FREUDENBERG K DIRSCHERL, W & EYER H (1931) *H ppe-Seyl Z* 202 128
FREUDENBERG K. & EYER, H (1932) *H ppe-S yl Z* 213 2 6
FREUDENBERG K. & WEGMAN T (1935) *H pp -Seyl Z* 233 159
GLENDENING M B GREENBERG D M & FRAENKEL-CONRAT H (1947) *J b l Chem* 167 125
HARINGTON C R & NEUBERGER A (1936) *B ochem J* 30 809
HARRIS J I & LI, C H. (1952) *J Amer chem Soc* 74 2945
H PKINS S J & WORMALL, A (1934) *B ochem J* 28 2125
JENSEN H & GEILING E. M K. (1928) *J Pharmacol* 33 511
JENSEN H & EVANS E. A Jr (1934) *Phy ol Rev* 14 188
KAPLAN E. H CAMPBELL L. D & MCLEA EN A D (1950) *B och m b phys Acta* 4 493
KAPLAN N O & GREENBERG D M (1944) *J b l Chem* 156 553
1 LENS J (1949) *B ch m b phys. Ac* 3 367
1 LI, C H (1954) *The Pr tei s* 2 Part A, 646 Academic Pres New Y k
2 LI, C. H. (1956) *Nat* 178 1402
1 MILLS, G L (1954) *B chem J* 56 230.
1 MOMMAERTS W F & NEURATH H (1950) *J b l Ch m* 185 909
3 N COL D S H W & SMITH L. F (195) *Bi hem J* 64 5 1
3 N COL, D S H W. Ph D thesis, U versity of Cambridge Ja uary 1958
3b NICOL, D S H W Ph D thesis *B ochem J* (1960) (in press)
4 PHILLIPS, D M P (1952) *B hem J* 50 479
5 PORTER R. R. (1953) *The Pr teins* 1 Part B 973 Academic Press New Y k.
6 REESE, H C T R F FRAENKEL-CONRAT H. & OLCOTT H S (1946) *J Amer Chem Soc* 68 1024
7 RYLE, A. P SANGER, F SMITH L F & KITAI R. (1955) *Biochem. J* 60, 541
8 SANGER F (1945) *Biochem J* 39 507
9 SANGER F (1949) *Ann Rep Chem Soc* 45 283
0 SANGE F & THOMPSON, E. O P (1953) *Biochem. J* 53 366
1 SCOTT D A (192) *J b l Chem* 63 641
2 SHONLE H A. & WALDO J H (1923 24) *J biol Chem* 58 731

43 SMITH E L, HILL R L & BORMAN A (1958) Biochim biophys Acta 29 207
44 STERN K G & WHITE A (1937) J biol Chem 117 95
45 STERN K G & WHITE A (1938) J biol Chem 122 371
46 DU VIGNEAUD V FITCH A PEKAREK E & LOCKWOOD W W (1931) J biol Chem 94 33
47 WITZEMANN E J & LIVSHIS L (19 3) J biol Chem 57 425

DISCUSSION

YOUNG I think it has been the hope of many people for many years that the existence of a prosthetic group of insulin might ultimately be revealed but since the work of Sanger and his colleagues has elucidated the complete chemical structure of insulin the hopes of finding a prosthetic group have tended to fade Dr Nicol s work done as it is with chemically purified fractions of insulin has in fact shown that the integrity of a number of parts of the molecule is essential for biological activity

This paper is now open for discussion

KRAHL Does Dr Nicol have any information about effect of reduction of the disulphide bonds in the A or B chains on activity of insulin?

NICOL I think a former colleague of ours at Cambridge Dr Ryle with Dr Sanger did some work on the reduction of these disulphide linkages but it was not quite clear from their work or the work of others in the United States and at Oxford which of the disulphide linkages definitely is the most important It seems that as soon as one of them goes activity drops suddenly if not entirely Perhaps someone else here might have some contribution on that topic

PETROW Willie has recently drawn attention to the fact that in these large polypeptide structures the actual units often are not important and that for example in the vasopressin type you can interchange amino acids It seems to me that with this work on insulin we are approaching the stage when it will be possible to synthesize an appropriate ring structure the actual amino acids in the ring will probably not be very important apart from certain amino acids of the type Dr Nicol has drawn attention to and then you would have to add the short chains to the sides I know that is looking ahead a good bit but I do think from what has been said this morning that we are actually getting near this point

RENOLD I should like to ask Dr Nicol what his prediction would be concerning the biological activity of insulin which has been iodinated I believe that this would be expected to alter the characteristics of the tyrosine groups?

NICOL Yes it would alter the tyrosine molecule With work on acetylation in covering of the hydroxyl groups the essential nature of the hydroxyl groups in that molecule has been shown Any alteration of activity which there may be in the iodinated insulin I should imagine would have to do with the essential nature of the aromatic amino-acid tyrosine

SCHAMBYE. Is the removal of zinc from the insulin molecule by versene nditional for the effect you obtained by the trypsin digestion or eration?

Secondly how completely was the zinc removed from the insulin olecule before you obtained the effect of trypsin?

NICOL. Without the removal of zinc trypsin does act on insulin It drolyses it to the extent of about 15 per cent of the total quantity of ptapeptides you would expect removed However you do not obtain ough of the DHA insulin to make separation easy When the zinc connt is reduced and it was reduced from about 0 5 per cent content to 025 per cent the extent of hydrolysis was more than doubled and ough DHA insulin was obtained to make the separation easier It may that the insulin molecule as such is composed of two of the units shown id zinc may combine both units through attachment perhaps to the stidine molecule as has been suggested perhaps the removal of the zinc iens the molecule more easily to the enzymic hydrolysis of trypsin but at is just a conjecture

Of course Dr Emil Smith of Salt Lake City Utah first showed that e action of leucine amino peptidase was increased on relatively zinc free sulin Our removal of zinc by versene was carried out by a method hich was first published in 1955 by Sluytermann (*Biochim biophys cta* 17 169)

BEHRENS You have reported that the separated A and B chains have low order of activity when tested on the rat diaphragm insulin assay lave you had an opportunity to study any other long chain polyeptides to see whether they share this property or is it unique for these agments of insulin? Is there any possibility that such insulin fragments ay be responsible for some of the insulin like effects on the rat diaphragm hat are observed in plasma?

NICOL We have not tested any long chain polypeptides of this nature ome different substances other than insulin or insulin related molecules f course give an increased glucose uptake The interest these two have or us is that one has to use such an unphysiologically high quantity of 5 micrograms to obtain an effect which you could get from insulin in our ands with about 0 1-5 micrograms of insulin

KORNER May I take issue with Dr Petrow on his suggestion that the mino-acid sequence of a protein is not a unique sequence for that protein nd that apart from a few important sites, the amino-acid composition s random?

Sanger has analysed insulin molecules from five different species and has hown that they differ from one another only in minor changes in aminocid sequence at one point in one of the peptide chains Ingram has demonstrated that the only difference in the haemoglobin molecules of

blood from normal people and from sickle-cell patients lies in a change of only one amino acid in over three hundred present in the molecule Yet this produces enormous changes in the chemical and physiological behaviour of the whole molecule These are but two examples from the large body of evidence accumulating and which shows that the amino-acid sequence of a protein is uniquely determined and is genetically controlled

PETROW Dr Korner broadly supports my view obviously because my point is that you have got certain sequences of amino acid which are very important attached to a ring which may itself have few prosthetic groups or important amino acids I believe what is required is a large ring of the right size and that there will be a fair amount of permutation possible with the unimportant amino acids in that ring

PART II

INSULIN AND TRANSPORT SYSTEMS

Chairman PROFESSOR F G YOUNG

THE MEMBRANE TRANSPORT OF SEVERAL MONOSACCHARIDES IN HEART MUSCLE AND THE REGULATION OF THIS PROCESS BY INSULIN ANTERIOR PITUITARY AND ADRENAL CORTICAL HORMONES*

H E. MORGAN E CADENAS AND C R PARK

Department of Physiology Vanderbilt University Medical School
Nashville Tennessee

In this paper we would like to report some observations on the passage of glucose and several other sugars through the cell membrane in muscle Two questions have been investigated first do sugars enter the cells by diffusion or by a specific transport system which requires interaction between the sugar and the membrane? Second to what extent can the hormonal control of glucose utilization be explained by regulation of the entry process? For such studies an *in vitro* muscle preparation is most desirable since close control of sugar and hormone levels is possible With these considerations in mind we have employed the isolated rat heart perfused through the coronary circulation by a technic analogous to that described by Bleehen and Fisher[1]

The particular advantages of the perfused heart compared to other *in vitro* preparations are first, sugar and hormones reach the cells rapidly by the intrinsic circulatory system of the muscle Second all substances enter the cell through the membrane itself since there are no cut edges Third the muscle remains in a good physiological condition as can be directly observed by the rate and force of the heart beat.

THE KINETICS OF THE TRANSPORT PROCESS

The penetration of a non metabolized sugar through the membrane has been estimated by the accumulation of the sugar within the cell after a suitable period of perfusion The accumulation has been measured by determining the total sugar content of the tissue and subtracting the fraction which is extracellular Labelled sor-

* This work was supported by grants from the National Science Foundation, the U S Public Health Service and the American Heart Association.
Part of this work has been presented earlier in preliminary form.

bitol an alcohol closely related in physical properties to glucose itself was used to measure the extracellular volume As shown in Fig 1 sorbitol distributes in a volume corresponding to 30 per cent of the tissue by weight This value is essentially constant after about 5 minutes up to periods as long as 2 hours as determined in recent

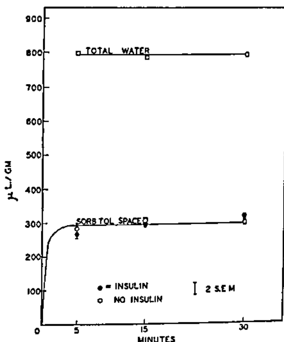


FIG 1 The distribution of sorb tol and the water content in the isolated perfused rat heart The curve has been drawn for the no insulin points 2 S E M is the abbreviation for tw ce the standard error of the mean

The heart was perfused with sorbitol 1-^{14}C and carrier to give a sorbitol concentr tion of 50 mg per cent in the perfusate The heart was extracted as described earlier[11] and the sorb tol content determined by estimation of radio ctivity using a liquid scintillation counter The sorbitol or extracellular volume was calculated as follows

$$\text{E C V } (\mu l/gm) = \frac{\text{Mean Conc Sorb in tissue } H_2O}{\text{Conc Sorb in perfusate}} \times \text{water content of tissue } (\mu l/gm.)$$

The amount of extr cellular sugar in any experiment was calculated by multiplying the E C V by the perfusate concentrat on of the sug r Intracellular sugar was determined as the total sugar minus the extracellular sugar

In these and all subsequent experiments to be described the perf sion medium was Krebs-Hensele t b carbonate buffer[6] gassed continuously with 95 per cent O_2 and 5 per cent CO_2 The temperature was maintained at 37

In these nd subsequent experiments in which hearts from normal rats were used the animals were fasted about 18 hours

experiments The distribution of sorbitol was measured in every experiment discussed below and was unchanged by hormonal or other experimental variables except for an increase of a few per cent when very high concentrations of sugar were added to the perfusion fluid

The rapid rate at which substances such as sugars exchange in the extracellular space of the heart preparation could be demonstrated by measuring the washout of labelled sorbitol After the extracellular water had first come into equilibrium with sorbitol during a period of 15 minutes (see Fig 1) the medium was then changed

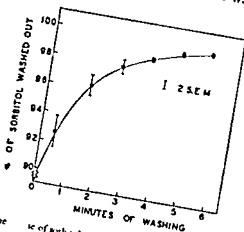


Fig. 2 The time [illegible] of sorbitol washout in the isolated perfused heart
See text for explanation

to sorbitol free buffer which was passed a single time through the heart and was collected during successive minute intervals for sorbitol analysis After 1 minute of this washing 93 per cent of the alcohol had been removed and after 5 minutes the washout was 99 per cent complete (see Fig 2)

The experiments of Fig 3 suggest that the entry of sugar into the cell does not occur by simple diffusion The intracellular accumulation of 3-o-methyl glucose a non metabolizable sugar was determined after a fixed period of perfusion at various perfusate concentrations The solid lines show that a non linear relationship of intracellular concentration to perfusate concentration was found in

both the control and insulin series If the penetration of the membrane occurred by simple diffusion it would be expected that the intracellular concentration would be directly proportional to the perfusate concentration It can be seen however, that the ratio of the intracellular to perfusate concentration decreased as the perfusate concentration increased the values falling from about 0 6 to 0 3 in the control and from 0 8 to 0 5 in the insulin series These kinetics suggest progressive saturation of combining sites for the sugar this would indicate that transport involves an interaction of the sugar

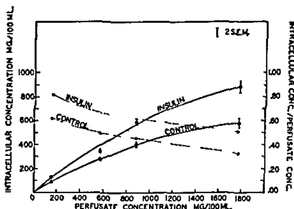


Fig 3 The relationship of intracellular accumulation of 3-o-methyl glucose to the concentration of the sugar in the medium

Hearts from normal rats were perfused in all cases for 30 minutes No glucose or other metabolizable substrate was present in the medium No visible impairment of function of the muscle has been noted under these conditions up to periods of 1 hour 3-o-methyl glucose was determined by the Nelson[9] procedure after extraction and deionization as described earlier [11]

with the membrane Similar observations have been made in the case of the erythrocyte and a discussion of their significance is to be found in a recent review by Bowyer [2]

More definitive evidence for interaction between the sugar and a membrane component was obtained by determining the presence of competition for transport between sugars No competition would be anticipated in a simple diffusion system In the control experiment at the top of Table I the intracellular accumulation of a non-metabolizable pentose L-arabinose is shown after perfusion for 15 minutes at a concentration of 500 mg per cent The addition

an equimolar concentration of 3-O-methyl glucose to the medium hibited arabinose penetration by about 86 per cent. Glucose was equally effective inhibitor. L-arabinose transport was accelerated insulin and the accelerated system could also be inhibited by ucose although the extent of inhibition apparently was not as eat. In the lower panel it can be seen that the penetration of ylose was also strongly inhibited by glucose.

TABLE I

THE INHIBITION OF L-ARABINOSE AND D-XYLOSE TRANSPORT BY 3-O-METHYL GLUCOSE AND D-GLUCOSE

L-arabinose was determined as described earlier.[11] The lack of L-arabinose and 3-O-methyl glucose utilization was determined in separate experiments in which measurable loss of sugar was observed when about 4 ml of medium were recirculated through the heart for 30 to 60 minutes.

Sugar added and molar ratio to L-arabinose	Insulin 3 μg/ml	Intracellular concentration of Pentose mg% ± S.E.M.
Perfusion for 15 minutes with medium containing 500 mg per cent L-arabinose with addition of other sugars as indicated		
None	0	157.1 ± 10.0 (16)
3-O-methyl glucose 1 : 1	0	21.8* ± 9.0 (7)
Glucose 1 : 1	0	38.0 ± 7.5 (8)
None	+	207.2 ± 6.6 (14)
Glucose 1 : 1	+	157.1* ± 19.1 (6)
Perfusion for 10 minutes with medium containing 400 mg per cent D-xylose with addition of other sugar as indicated		
None	0	123.0 ± 12.0 (6)
Glucose 1.25 : 1	0	40.6 ± 11.0 (6)

* P < .005 vs. appropriate control

Table II shows conversely that glucose transport can be inhibited by L-arabinose and 3-O-methyl glucose. In the absence of insulin the transport of glucose is indicated by the utilization of the sugar from the medium. Despite appreciable transport into the cell it can be seen that the free glucose distribution remained less than the sorbitol or extracellular space of 300 μl/gm. As discussed in detail in earlier papers[11,12,14] this indicates that the glucokinase system

keeps pace with glucose entry under these conditions With the addition of insulin however transport was greatly increased as shown by the rise in utilization and now the free glucose space considerably exceeded the sorbitol space This can be most readily interpreted as due to an acceleration of transport to the point where the entry of glucose is so rapid that it exceeds the capacity of the glucokinase system The next lines of the table show that L-arabinose was a poor inhibitor of glucose transport a 4 1 molar ratio of pentose to glucose did not appreciably affect utilization and even a

TABLE II

THE INHIBITION OF GLUCOSE TRANSPORT BY L ARABINOSE AND 3-O-METHYL GLUCOSE

In all experiments glucose was perfused at a concentration of 150 mg per cent for 15 minutes Glucose was measured as previously described[11] using the hexokinase-zwischenferment method to ensure specificity

Sugar added and molar ratio to glucose	*Insulin 0 3 µg /ml*	*Utilization mg /g /hr ± SEM*	*Glucose space µl /g ± SEM*
None	0	4 5 ± 0 5 (10)	227 ± 9
None	+	9 6* ± 0 5 (15)	458* ± 14
L-arabinose 4 1	+	9 7 ± 1 0 (10)	470 ± 14
L-arabinose 12 1	+	7 4* ± 0 6 (11)	385* ± 12
3-o-methylglucose 4 1	+	5 6* ± 1 3 (5)	275* ± 17
3-o-methylglucose 8 1	+	4 6* ± 1 0 (5)	272* ± 5

* P < 01 vs appropriate control

12 1 ratio was not strongly inhibitory 3-o-methyl glucose on the other hand inhibited strongly at 4 and 8 1 molar ratios In all instances where utilization was reduced the glucose space was also reduced This is evidence that competition takes place at the transport step since inhibition of metabolism within the cell should cause an increase in space due to a rise in intracellular free glucose

In earlier studies with the erythrocyte it was possible to show that competition between sugars can lead under certain conditions to transport of one of the sugars against a concentration gradient[13] This active transport could also be shown in the case of the heart

nuscle (Fig 4) When the tissue was perfused with 150 mg per ent of 3-o-methyl glucose the intracellular concentration rose to bout 86 mg per cent in the first 5 minutes and then increased lowly to about 93 mg per cent during the next -5 minutes However when glucose was added to the medium at the 5-minute point he intracellular 3-o-methyl glucose decreased the extent of fall being dependent on the amount of glucose added

With the addition of a tenfold concentration of glucose the intracellular 3-o-methyl glucose was reduced to 34 mg per cent that is

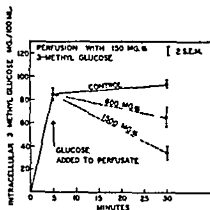


Fig 4 The active tran port of 3-o-meth yl glucose out of the cell due to competition by glucose for the tra port system

The concentration of 3-o-methyl glucose in the perfusate was maintained at 150 mg per cent throughout the experiment The concentration of glucose added is indicated by the values above the broken lines.

to 40 per cent of the control value Since 3-o-methyl glucose could not be metabolized it must have been transported out of the cell although this movement was against the concentration gradient This can be most easily explained by depression of 3-o-methyl glucose influx due to the competitive activity of glucose for the transport system at the external surface of the membrane Inside the cell however the glucose was rapidly metabolized and was therefore less effective as a competitor for the efflux process As a result efflux transiently exceeded influx and a net movement took place of 3-o-methyl glucose out of the cell A competition effect of this

kind is readily explained on the basis of a carrier sugar transport and is difficult to account for by oth (see discussion by Bowyer[2])

On the basis of these competitive studies the ord the various sugars for the membrane-combining site glucose > 3-o-methyl glucose ≫ xylose and L- demonstration in these competition studies that non-metabolizable sugars employ the same transport also provides valuable support for the assumption n authors that transport data obtained with these sugars can be used to draw conclusions regarding the glucose itself In regard to the effect of insulin it may that although the hormone accelerates transport it does the system to one of simple diffusion since the kinetics saturation type and competition between sugars is still d The absence of an added diffusion element in the insulinized is also indicated by the failure of sorbitol to enter the cell (see although this substance is very similar to glucose with size and lipid solubility

The present observations are in harmony with the earlier of Fisher *et al*[34] showing that galactose penetration in the heart does not conform to the kinetics of simple diffusion and galactose and glucose are competitive for the transport process

THE EFFECT OF VARIOUS HORMONAL FACTORS ON MEMBRANE TRANS

While the acceleratory effect of insulin on sugar transport to be well established from work in many laboratories very is known regarding the possible inhibitory effects of other on the transport process although such effects would from earlier studies of glucose uptake Preliminary presence of transport inhibitors in the muscle from alloxan-diabetic and also from normal animals is presented in Table III Since glucose which is rapidly metabolized was used as the test sugar it was necessary to evaluate transport rates indirectly by measurement of the utilization and distribution of the sugar as discussed below Data obtained with hearts from normal animals with and without insulin are shown for reference on the first two lines The glucose utilization by the heart from alloxan-diabetic rats was severely depressed the glucose distribution however remained approximately equal to the sorbitol space This indicated that the inhibition of utilization

as at the transport step since if hexokinase had been inhibited e glucose should have accumulated within the cell.

These tentative conclusions could be supported and extended ing a more direct and sensitive experimental method in which easurement was made of the transport of a non metabolizable gar out of the cell. The technic was briefly as follows: the heart as first perfused with a high concentration of L-arabinose in order accumulate a large amount inside the cell. The medium was then anged to sugar-free buffer which passed a single time through the art and was collected over successive intervals of 1 or 2 minutes.

TABLE III

THE EFFECT OF INSULIN AND ALLOXAN DIABETES ON GLUCOSE TRANSPORT IN THE ISOLATED RAT HEART

The number of experiments is shown in parentheses. The asterisk denotes p value of <0.01 with reference to the normal control without insulin.

Alloxan was injected where indicated in a dosage of 60 mg/kg i.v. 48 hours before sacrifice. Food was withheld during the last 24 hours and the terminal blood sugar concentrations were uniformly above 300 mg per cent.

Type of animal	*Insulin units/ml*	*Utilization mg/g/hr ± S.E.M.*	*Glucose space µl/g ± S.E.M.*
Normal	0	4.3 ± 0.5 (10)	227 ± 9
Normal	1×10^{-1}	9.6 ± 0.5 (15)	435 ± 14
Alloxan diabetic	0	0.7 ± 0.2 (11)	277 ± 9

P < .01 vs Normal Animal – No Ins.

he amount of pentose appearing in the perfusate under these conitions could be used to measure transport out of the cell provided xtracellular arabinose was first removed. The collection of perusate during the first 5 minutes was therefore discarded since this ime was sufficient to remove all extracellular sugar as judged by the washout of radioactive sorbitol (see Fig. –). The greater sensitivity f this method can probably be ascribed to the fact that transport nder these conditions is essentially uni-directional since the continuous washing prevents accumulation of extracellular sugar and minimizes back transport into the cell.

In Fig. 5 some of the results obtained are shown. Hypophysectomy ed to a marked rise in transport as compared to the normal animal

Alloxan diabetes caused a marked depression but of insulin *in vitro* the rate could be restored to the high value of the normal In the diabetic insulin ' increase in rate demonstrating that a very broad rate is under hormonal regulation The effect of ins from hypophysectomized animals was relatively

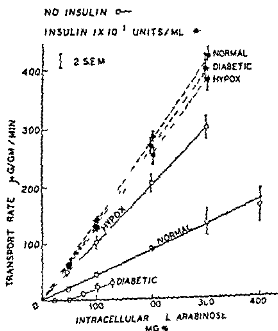


Fig 5 The effect of alloxan diabetes and hypophysectomy on the transport of L ar out of the cells of the isolated heart

The technic is described in the text The concentration of intracellular L-arabin calculated by su nming the pentose contents of the perfusat collections and the pentose of the tissue at the end of the experiment The intracellul r L-arabinose concer was cal ulated on th a sumption of a uniform distribution w thin the cell.

sumably to the already high control rate Although the rates out insulin were widely separated in the three types of prepara the rates with insulin were surprisingly close It was also worthy that in any given series the rates showed remarkable stancy as judged by the standard error of the mean

Fig 6 shows the effect of various concentrations of insulin a

ring the preliminary period of pentose accumulation and continued during washout of the sugar 1×10^{-4} units per millilitre produced a large increase in rate amounting to 78 per cent of maximal represented by the value for 1×10^{-1} units/ml 1×10^{-5} and 1×10^{-6} units/ml did not significantly stimulate the rate under the conditions tested This sensitivity appears to be about the same as noted in other preparations such as the isolated rat diaphragm

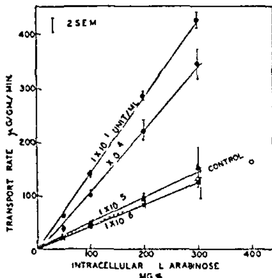


Fig 6 The effect of insulin concentration on the rate of outward transport of L arabinose The procedure is outlined in the text The standard errors for the control rate are shown in

The outward transport technic has also made it possible to determine with some precision the time of onset of insulin activity and to follow the course of acceleration minute by minute As seen in Fig 7 when insulin was added to the perfusate during outward transport of sugar an effect was noticed within about 2 minutes. The onset was equally quick in the diabetic muscle but the subsequent acceleration was much slower Even though the insulin added was 1000 times the minimal effective concentration it took two to four times longer in the diabetic to reach the maximum rate

This is a manifestation of the insulin resistance which has been frequently noted in muscle from alloxan-diabetic animals

It is of course most important to know what hormonal factors are responsible for the inhibition of transport in the diabetic state On the basis of a large background of knowledge from many sources it would be anticipated that growth hormone and adrenal steroids might be involved Fig 8 shows the effect on transport of

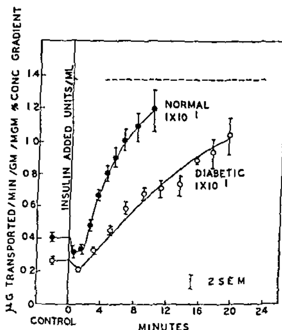


Fig 7 The time course of the insulin effect in he rts from normal and alloxan-diabetic rats Insulin w s ad led in a concentration of 0 1 units per ml at 0 time The horizontal broken l e indicates the maximum rate obtained with insulin as determined in the experiments of Fg 5

daily injections of growth hormone for 5 days and/or cortisone into hypophysectomized animals The rate was moderately reduced by either growth hormone or hydrocortisone alone and the combination of both hormones caused a severe depression of transport to the diabetic level The observations suggest that a part of the well-known diabetogenic activity of growth hormone and cortisone may be attributed to inhibition of glucose transport in muscle It should be noted however in the case of cortisone alone that very high

ses were employed. It remains to determine the effect of cortisone lower levels since earlier studies of glucose utilization by muscle om hypophysectomized animals (Park *et al.*[10]) have not shown renal steroids to be effective inhibitors in the absence of the uitary. In regard to growth hormone, we have been unable to plicate the interesting report of Bronk and Fisher[3] despite re-

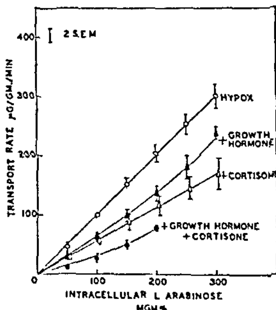


Fig. 8. The effect of growth hormone and cortisone in vivo on the transport of L-arabinose in the isolated hearts from hypophysectomized rat.
Highly purified growth hormone, gift of the Endocrinology Study Section of the National Institutes of Health, was injected daily in a dosage of 0.1 mg. per 100 g. per day. The daily dosage of cortisone was about 2.5 mg. per 100 g. per day. Treatment was continued for 5 days; the last injections were made about 18 hours before sacrifice.

peated efforts that growth hormone added *in vitro* inhibits glucose transport and utilization. Experiments carried out by Dr Margaret Henderson in this laboratory using a very active growth-hormone preparation and also the same material (B 168) employed by Bronk and Fisher have shown slight acceleration of transport in hearts from normal animals, and a large insulin-like acceleratory effect in hearts from hypophysectomized animals.

Concluding Remarks and Summary

The regulation of transport appears to be the closest approach that we can make at present to defining the site of action of the above hormones on glucose uptake by muscle It would not be proper however to assume that transport is necessarily the primary point of action of insulin or the other factors The recent studies of Randle and Smith[15] for example illustrate that sugar permeability can be strongly influenced by changes in intracellular metabolism and a number of insulin effects on amino-acid and acetate metabolism discussed in this symposium and elsewhere are not explained at present by any single point of action

The principal conclusions which we would like to draw from the present position of our work are as follows the transport of D-glucose D-3-o-methyl glucose L-arabinose and D-xylose through the cell membrane in the presence or absence of insulin involves interaction of the sugar with a specific component of the membrane The membrane-combining site shows substrate stereo-specificity and is shared by pentoses and hexoses Glucose and 3-o-methyl glucose have high affinities relative to arabinose and xylose The membrane-transport process can be regarded as the first step in the metabolism of sugar by muscle it antecedes and is distinct from phosphorylation by the hexokinase system and the product of transport is the free sugar In muscle transport is ordinarily slow and appears to limit the rate of glucose uptake As the rate-limiting process it is an appropriate site for hormonal regulation Insulin causes a striking acceleration of transport and alloxan diabetes a marked inhibition The inhibition appears to be due to the effects of pituitary growth and adrenal steroids in conjunction with a lack of *insulin*

Subsequent experiments using hearts from hypophysectomized rats have shown that the transport rates vary from those reported here to rates equal to or less than those of normal animals The explanation of this variation is uncertain but may be related to *variations in endogenous insulin levels to which the animals are* unusually sensitive Several mechanisms have been considered by which the *in vivo* injection of growth hormone and cortisone could result in lower transport rates The hormones could (1) directly inhibit transport or (2) decrease the sensitivity of the tissue to endogenous insulin At present we would favour the second

possibility For additional discussion of these questions see Morgan H E. Henderson M J Regen, D M and Park, C R. *Ann N Y Acad Sci* 82 387 1959

REFERENCES

1 Bleehen N M. & Fisher R. B (1954) *J Physiol* 123 260.
2 Dowben F (1957) *Int Rev Cytology* 6 469
3 Bronk M Fisher R. B (1957) *J Physiol* 136 435
4 Fisher, R. B & Lindsay D B (1956) *J Physiol* 131 526
5 Krebs, H. A. & Henseleit K (193) *Z Physiol Chem* 210 33
6 Morgan H E., Henderson M J Regen D M. & Park, C. R. To be published.
7 Morgan H. E. & Park, C. R. (1958) *Fed Proc* 17 1099
8 Morgan H E. Cadenas E. & Park C R. (1958) Abstracts IV *International Congress Biochem* 9-14
9 Nelson N (1944) *J biol Chem* 153 375
10 Park, C R Brown D H Cornblath, M Daughaday W H & Krahl, M. E. (1952) *J biol Chem* 197 151
11 Park, C. R., Bornstein J & Post R L. (1955) *Amer J Physiol* 182 12
12 Park C. R & Johnson L. H. (1955) *Amer J Physiol* 182 17
13 Park, C R., Post R. L., Kalman C F Wright J H. Jr Johnson L. H. & Morgan H E. (1956) Ciba Foundation Coll. Endocrinol. 9 240.
14 Park C. R., Johnson L. H Wright J H Jr & Batsel H (1957) *Amer J Physiol* 191 13
15 Randle P J & Smith J This Symposium.

INSULIN AND THE TRANSPORT OF SUGARS

R. B. Fisher

Department of Biochemistry Oxford

The perfused heart preparation has given insight into some of the problems associated with the permeability hypothesis of insulin action which are not easily attacked by the use of the whole or the eviscerated animal or by the use of the isolated diaphragm. This paper deals with some of these problems

The perfused preparation is the most reliable one available for the investigation of the insulin-dependence of the penetration of sugar into cells As Lindsay and I have shown (Fisher and Lindsay [illegible] the penetration of galactose into the heart is markedly depressed in the presence of glucose in the perfusate This is under[illegible] both sugars are competing for a common carrier [illegible] penetration of sugars with a lower affinity than [illegible] carrier would be expected to be correspondingly [illegible] depressed by glucose *As a consequence* [illegible] when the sugar to be studied is introduced [illegible] tains glucose the rate at which the sugar under [illegible] cells will not be a true measure of its intrinsic [illegible] but will be much more an index of its ability [illegible] glucose for the common carrier Furthermore [illegible] intracellular glucose concentration it will [illegible] which glucose can compete for the carrier This [illegible] any insulin effect on the rate of penetration of the [illegible] The scale of the insulin effect could be so fa[illegible] factors that it might be too small to be demonstra[illegible]

We have made one test of these rather theo[illegible] Sacks and Bakshy[2] showed that insulin increases L-[illegible] tion into the cat heart in [illegible] but has no determ[illegible] penetration of D-arabinose Dr Zachariah has [illegible] sugars in the perfused rat heart in my laboratory a[illegible] to glucose-free perfusat[illegible] L-arabinose penetra[illegible] as does galactose *the intracellular concentration* [illegible] external concentration in 10-15 minutes Its ra[illegible]

already been shown by Sacks to be increased by insulin D-arabinose penetrates much more slowly the intracellular concentration reaches only about one-third of the extracellular concentration after an hour But the rate of penetration of D-arabinose is markedly increased by insulin We hope to make competition experiments in order to determine whether the discrepancy between our finding and that of Sacks is due to the sort of factors which I have discussed

One might perhaps have made this sort of test with the isolated diaphragm as indeed some workers have done but there are grounds for believing that the diaphragm does not yield useful indications of the kinetics of penetration This is illustrated by our experience of the study of the kinetics of efflux of galactose from the heart in experiments of the same general nature as those described by Dr Park Mr D A B Young has made experiments in which the heart is first perfused with galactose and any desired concentration of insulin and is then transferred to a second apparatus in which the perfusate contains the same concentration of insulin but no galactose After washing out the dead space effluent from the heart is collected in timed periods By virtue of the high rate of perfusion the galactose concentration in the effluent is always very low so that we are effectively studying the efflux of galactose into galactose-free medium

The data do not conform to a first order process When the appropriate semilogarithmic plot is made the lines which are obtained always make positive intercepts on the ordinate axis The data do however conform with one qualification to the much more complex equation for efflux from cylinders within which there is a uniformly low diffusion coefficient The expected relation which is given by Crank[5] is shown in Fig 1 In this figure the fraction of the intracellular solute extracted in time t is plotted against Dt/a^2 where a is the radius of the cylinder and D is the diffusion coefficient within it

We have transformed our observed fractions into the corresponding values of Dt/a^2 with the help of this curve and tested the fit to the curve by plotting these derived values of Dt/a^2 against time We should expect straight lines through the origin provided that the fluid which we were collecting came immediately from the surfaces of the cells Since exchange between interstitial fluid and perfusate is interposed between the cell surface and our collecting vessels we can expect the experimental curve to deviate to some

extent from a straight line through the origin. We have made a theoretical study of the effect of superimposing an exponential exchange between tissue fluid and perfusate on the Crank relation. We *find that if the time-constant of the exponential process is* k then the plot obtained should be a straight line for all times greater than $3/2k$ and that this line should meet the time-axis at $t = 1/k$. This theoretical expectation is shown in Fig. 2. Our experimental data fit well with this, as is shown in Fig. 3. Data obtained without

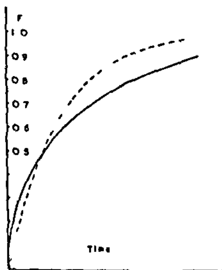


Fig. 1 The expected time-course of equilibration of an infinite volume of solution with a cylinder within which the diffusion coefficient is uniformly low. The interrupted curve represents a first-order equilibration such that the time for 50 per cent equilibration is the same as for the process involving uniformly slow diffusion through the cylinder.

insulin and with a wide range of insulin concentrations is shown in this figure, and it will be seen that despite a great variation in the slopes of the lines they cut the time-axis in general in the region of 0.5 minutes. The first points on the lines are therefore on the linear portions of the curves and we can interpret the slopes of these lines as measures of D/a^2.

The intercepts on the time-axis indicate a half time of exchange between tissue fluid and perfusate of 0.3–0.4 minutes. Despite this

already been shown by Sacks to be increased by insulin D-arabinose penetrates much more slowly the intracellular concentration reaches only about one-third of the extracellular concentration after an hour But the rate of penetration of D-arabinose is markedly increased by insulin We hope to make competition experiments in order to determine whether the discrepancy between our finding and that of Sacks is due to the sort of factors which I have discussed

One might perhaps have made this sort of test with the isolated diaphragm as indeed some workers have done but there are grounds for believing that the diaphragm does not yield useful indications of the kinetics of penetration This is illustrated by our experience of the study of the kinetics of efflux of galactose from the heart in experiments of the same general nature as those described by Dr Park Mr D A B Young has made experiments in which the heart is first perfused with galactose and any desired concentration of insulin and is then transferred to a second apparatus in which the perfusate contains the same concentration of insulin but no galactose After washing out the dead space effluent from the heart is collected in timed periods By virtue of the high rate of perfusion the galactose concentration in the effluent is always very low so that we are effectively studying the efflux of galactose into galactose free medium

The data do not conform to a first order process When the appropriate semilogarithmic plot is made the lines which are obtained always make positive intercepts on the ordinate axis The data do however conform with one qualification to the much more complex equation for efflux from cylinders within which there is a uniformly low diffusion coefficient The expected relation which is given by Crank[5] is shown in Fig 1 In this figure the fraction of the intracellular solute extracted in time t is plotted against Dt/a^2 where a is the radius of the cylinder and D is the diffusion coefficient within it

We have transformed our observed fractions into the corresponding values of Dt/a^2 with the help of this curve and tested the fit to the curve by plotting these derived values of Dt/a^2 against time We should expect straight lines through the origin provided that the fluid which we were collecting came immediately from the surfaces of the cells Since exchange between interstitial fluid and perfusate is interposed between the cell surface and our collecting vessels we can expect the experimental curve to deviate to some

fficient exchange between cells and medium in the perfused heart he kinetics of cell penetration are still obscured to some extent by xtracellular exchange processes This sort of obscuration must be much more marked in the isolated diaphragm Not only is the ime-course of exchange between medium and extracellular fluid much slower but the cells of the tissue must be non uniformly exposed to the sugar being studied for a large part of the period of observation Judging from Kipnis and Cori s[7] data for thiosulphate he extracellular concentration of solutes entering the diaphragm from the external medium is not likely to become stable until more han 30 minutes have passed, even for small molecules with something of the size of insulin the delay may be expected to be much longer Probably therefore one is dealing over a large fraction of the period of observation with a preparation in which the cells are not only non-uniformly exposed to the sugar but even more non uniformly exposed to insulin

Reverting to our data for the efflux of galactose from the heart we can now consider the significance of the values of D/a^2 which we obtain from the slopes of the straight lines in Fig 3 Since a is the radius of the heart cell which can be assumed to be constant these variations imply variations in D the apparent diffusion coefficient of galactose in the cell This apparent diffusion coefficient will be the product of the true diffusion coefficient of the galactose-carrier complex and of the ratio of galactose-carrier complex concentration to free galactose concentration Since the diffusion coefficient of the complex is unlikely to be variable the variation in apparent diffusion coefficient may be taken as a measure of the variation in concentration of total available carrier We have therefore made some preliminary explorations of theories of insulin action on cell permeability on the assumption that the concentration of available carrier is altered by insulin and is directly proportional to the experimentally determined value of $D\,a^2$

In the first place we have tested the hypothesis suggested by Fisher and Lindsay[8] that the carrier (C) combines with an inhibitor (X) to form an inactive complex (CX) and that insulin also combines with (X) to form a complex (IX) thus freeing carrier This leads to an equation with four unknowns We have fitted data for controls and for four insulin concentrations to this hypothesis by the method of least squares and found that the resulting equation fits all the points remarkably well This is shown in Fig 4

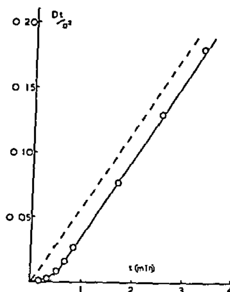


FIG 2 The effect of interposition of an exponential exchange between cells and perfusate on the plot of Dt/a against t

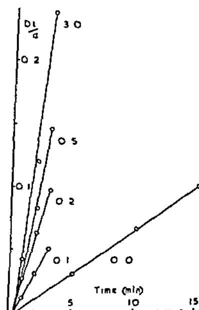


FIG 3 The experimentally determined relation between Dt/a and t The numbers against the lines represent the concentrations of insulin used in unit /litre

e presence of insulin is a measure of the insulin-dependent carrier We have also supposed that the insulin-dependent carrier arises by mbination of insulin with some cell constituent present in limited nount so that there is a Michaelis-Menten type of relation between sulin concentration and increase in apparent diffusion coefficient er the control value This hypothesis is easy to fit to the data by andard methods and as Fig 5 shows it fits just as well as the first pothesis The two best fit curves shown in Figs 4 and 5 are arcely distinguishable

It ought however to be possible to distinguish between the potheses since one involves affinity of the sugar studied for a ngle carrier and the other its affinity for two separate carriers and is highly unlikely that the affinities for two independent carriers f two or more different sugars will run in parallel Repetition of e work with other sugars is therefore planned Another approach suggested by the work of Bronk and Fisher[2] on the effect of rowth hormone preparations on the permeability of the heart to alactose This work suggested that a purified beef growth hormone reparation approximately halved the apparent diffusion coefficient f galactose into the heart both in the presence of and in the absence f insulin This is much more easily explained on the single carrier ypothesis

At present we incline towards the single carrier hypothesis, since he dual carrier hypothesis is not needed to account for penetration n the absence of insulin and since the growth hormone observations re more readily accounted for by the single carrier hypothesis.

Work of this kind cannot naturally contribute much to the mportant question of the ability of the permeability effect of nsulin to account for all its physiological activity But very few of its effects have been claimed to be totally inexplicable as secondary onsequences of the permeability effect The latest of these is the ffect on the carbohydrate metabolic pattern of the isolated diaphragm which has been demonstrated so elegantly by Chain *et al*[4] would like to conclude by offering an explanation of this effect in erms of the permeability effect

It is clear from the work of Candela *et al*[3] Zierler *et al*[10] and Zierler[11] that enzymes are continually escaping from the cells of he usual kind of diaphragm preparation. Escape will certainly occur from damaged cells but Zierler believes that he has evidence that most of this loss occurs across the intact cell membrane If this is so

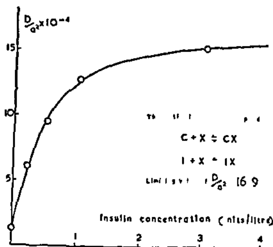


Fig 4 The fit of the experimental data to the single carrier hypothesis of insulin action.

We have also tested an alternative hypothesis namely that there are two carriers one insulin-independent and one insulin-dependent as suggested by Resnick and Hechter [8] To do this we have supposed that the apparent diffusion coefficient in the absence of insulin is a measure of the activity of the insulin-independent carrier and that the difference between this and the apparent diffusion coefficient in

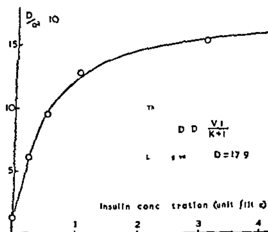


Fig 5 The fit of the experimental data to the dual carrier hypothesis of insulin action

it will be evident from what has been said about diffusion in the diaphragm that enzyme concentrations in the interstitial fluid will much exceed those in the medium It is therefore well within the bounds of possibility that the interstitial fluid may constitute a qu‹ active kind of Meyerhof muscle extract (One would expect that the enzymes most readily lost from muscle cells would constitute a glycolytic system in view of the known association of oxidative systems with the particulate fraction of the cell) In the absence of insulin when the extracellular glucose concentration should be much higher than the intracellular concentration this would mean that glucose metabolism would be primarily glycolytic though of course the pattern would not necessarily mimic exactly that of glycolysis in muscle cells In the presence of insulin when glucose can pass readily into cells the pattern would be expected to shift towards oxidative and anabolic pathways Some support for this is to be found in earlier data of Beloff-Chain *et al*[1] which suggest that in the absence of insulin most of the glucose in the diaphragm is extracellular

I believe therefore that there is as yet no need to move from th position that the permeability effect of insulin is in all probability responsible for its physiological effects

REFERENCES

1 BELOFF-CHAIN A CATANZARO R CHAIN E. B MASI I POCCHIARI F & RO SI C (1955) *Proc Roy Soc* B 143 481
2 BRONK M S & FISHER, R B (1957) *J Pl ysiol* 136 435
3 CANDELA R R SOLS E ALVARADO R. SANTIAGO E VILLAR PALASI, C. & CANDELA J L R (1957) *B ll S c Chim e B ol* 39 Suppl II 67
4 CHAIN E B BELOFF-CHAIN A. & POCCHIARI F (19 6) *Selected Sc ent fic Papers fr m the I t tuto Superio e d San ta* 1 393
5 CRANK J (1956) *Clarendon Press* p 72
6 FISHER R B & LINDSAY D B (1955) *J Phys l* 131 5 6
7 KIPNIS D M & CORI C F (1957) *J b ol Chem* 224 681
8 PESNICK O & HECHTER O (1957) *J b ol Cl em* 224 941
9 SACKS J & BAKSHY S (1957) *Amer J Pl ysiol* 189 339
10 ZIERLER K L LEVY R I & ANDRES R (1953) *Johns H pk Ho p B ll* 92 7
11 ZIERLER K L (1956) *Amer J Phys ol* 185 1

DISCUSSION

YOUNG The earlier studies of Hober Loewi Pollak and others have indeed fallen on most fruitful ground in recent years and I think Dr Park s contribution is a most interesting and important one in this connection It particularly appeals to me that he is able so elegantly to make use of

the outward transport of sugars from cells in order to study the various hormonal effects

I would like to ask Dr Park a question myself – you mention in your abstract that you have found either no effect of growth hormone *in vitro* on glucose transport or an acceleratory effect Do you find this acceleratory effect in hearts from normal rats or in hearts from hypophysectomized rats, or in both sorts of heart

PARK In the heart from normal rats we have not observed any significant effect of growth hormone *in vitro* on transport In hearts from hypophysectomized rats there is a marked acceleratory effect

LEVINE In connection with Dr Park's experiments on outward transport I should like to tell him that Dr Goldstein has confirmed his findings using the eviscerated nephrectomized dog This phenomenon which Dr Park demonstrated in the perfused heart obtains in the whole eviscerated animal which must include of course skeletal muscle and other insulin-sensitive tissues

Some years ago Dr Goldstein and I injected cortisone into intact dogs in high amounts until the animals became insulin insensitive Then we eviscerated them and studied the distribution of galactose with and without insulin We could not, and cannot at present demonstrate any inhibition of insulin action in the eviscerated animal I am wondering therefore whether we did not use a sufficient amount of cortisone to demonstrate an inhibition or whether the inhibitory effect is not present in all tissues. In the whole animal where the mass of the heart of course is small in comparison with the muscle and fat tissues, the inhibition phenomenon may not become significant This would be important to resolve One could draw the conclusion that this inhibition phenomenon has a relation to the diabetogenic effect in the whole organism and it is not yet clear whether it does or not

SMITH I am sure we would all agree with Dr Fisher that there are limitations to the use of diaphragm in studies of the transport of sugars There is however one point in his discussion on which I would like to comment

In the work which Dr Randle and I describe in our own paper (see p 65) we were indeed aware of reports that certain enzymes are believed to leak from the hemidiaphragm during incubation but we could not show that these would utilize free glucose We incubated a number of hemidiaphragms in bicarbonate medium for 30 minutes under our usual conditions then with fresh portions of the medium and incubated these again for a further period of 1 hour under the same conditions but in the absence of tissue no further change of glucose content of the medium occurred after separation from the tissue Undoubtedly protein is released into the medium by the incubated hemidiaphragm – mainly from

the cut ends of the fibres I imagine – and this material may well include certain of the enzymes concerned in the utilization of glucose But it seems from this experiment that under our conditions at any rate no dissimulation of glucose itself occurs in the medium bathing the tissue Of course we do appreciate that this does not exclude the possibility of some glucose being metabolized in the extracellular compartment of diaphragm by enzymes bound to the surface of the cell as Shaw and Stadie have recently suggested

STEWART Keyl and Dragstedt have shown that the embyronic chick heart 3 days old is refractory to the toxic action of cardiac glycosides and that at the tenth day the heart becomes sensitive to the glycosides it is at this later stage that the pancreas begins to function We have found in the guinea-pig pre-treated with insulin that the toxicity of the glycosides digoxin digitoxin and ouabain is markedly enhanced and I wonder whether Dr Park or Dr Fisher have done any work on sugars such as digitoxose to know whether the transport of them is markedly accelerated by insulin or whether any other sugars linked to other substances to form glycosides are also rapidly taken up

FISHER I do not know whether Dr Park and I ought to come here as twins and say No together but certainly for myself I have not and it is a very interesting idea

Whilst I am here I would just like to say something about this business of the growth-hormone effect I think a possible explanation is that when we measured the effect of our Wilhelmi beef growth-hormone preparations on the penetration of galactose into the heart we had to have glucose present in the perfusate to slow the penetration down to manageable dimensions and it is quite conceivable that the effect that we obtained there is secondary to the effect on glucose metabolism

D ARCY I would like if I may to direct my question to Dr Park I was very interested to hear Dr Park mention that hypophysectomy increases the transport rate whereas growth hormone and cortisone *in vivo* depresses it I would like to know for information if you have tried the effects of corticotrophin either *in vivo* or *in vitro* preparations

PARK We have not tested this at all

D ARCY I have wondered if that may possibly in some way explain the difference between your *in vivo* effects with growth hormone and your opposite results which you found *in vitro* Could it be contamination of your growth-hormone preparation say with another trophic hormone from the pituitary?

PARK Contamination by other [illegible] a possibility and insulin-like or hypoglycaemic acti[illegible]rved with pituitary factors o[illegible]
insulin like ac[illegible] o or in [illegible]

ormone preparations in many laboratories and efforts to separate this tivity by chemical means from growth activity have not been successful our hands

BESSMAN I should like to ask whether the kinetics of sugar transport ight be equally referrable to an energy mechanism. Wouldn t the uations fit just as well? There may be an energy mechanism which sulin enhances or which insulin inhibits and it may not be necessary refer all the thinking merely to a membrane — I apologize for the word nerely to this very mysterious thing called a membrane It might be ore fruitful to compare the different acceptor systems for energy in the arious tissues We find the heart different from the diaphragm and also om the skeletal muscle We also find all the tissues different from the rain and it is probable that the various energy transport mechanisms or nergy-developing mechanisms differ in the various organs particularly the anatomic arrangement of the enzymes involved

We have proposed a hypothesis which assigns the function to insulin f the connection of hexokinase to the energy-generating site of mitohondria Under these circumstances we might possibly say that the ools which were mentioned of different concentrations of glucose ould well be different areas in the cell (as we can see on electron microcopy) with the membranes within the cell sequestering the mitochondria f an enzyme system were attracted to or attached to a mitochondrion endering that area of the cell more effective it would produce a different ool of utilization of sugar and generation of energy

KRAHL. Dr Hewson Swift and I have electron micrographs of diahragm which show a relatively thick layer of mesothelial tissue on both he pleural and peritoneal sides of the true diaphragm muscle It may be hat these layers are related to the differences in kinetics of entry of various ugars and to the differences between diaphragm and heart muscle in their responses to insulin

LITROW Just a very small point it has been shown for example by Dr Nicholls at King s, that the effect of cortisone on the reticulo-endothelial system is antagonized by oestrogens We know that cortisone is diabetogenic if anything whereas oestrogens have the opposite effect in the Houssay animal and I wondered whether you had tried the effect of oestrogens in antagonizing the effect of the cortisone The second point is that in discussing these effects in animals we have to remember that the adrenal corticoids do not start being produced normally in the animal immediately at birth they only start being produced immediately afterwards, and that the nature of the hormones produced by each animal is different. For example the rat produces I think a lot more corticosterone than cortisone so I think we have a very complicated picture there

PARK We have done nothing at all with the oestrogens I would say

the cut ends of the fibres I imagine – and this material may well *include* certain of the enzymes concerned in the utilization of glucose But it seems from this experiment that under our conditions at any rate no dissimilation of glucose itself occurs in the medium bathing the tissue Of course we do appreciate that this does not exclude the possibility of some glucose being *metabolized in the extracellular* compartment of diaphragm by enzymes bound to the surface of the cell as Shaw and Stadie have recently suggested

STEWART Keyl and Dragstedt have shown that the embyronic chick heart 3 days old is refractory to the toxic action of cardiac glycosides and *that at the tenth day* the heart becomes sensitive to the glycosides it is at this later stage that the pancreas begins to function We have found in the guinea pig pre treated with insulin that the toxicity of the glycosides digoxin digitoxin and ouabain is markedly enhanced and I wonder whether Dr Park or Dr Fisher have done any work on sugars such as digitoxose to know whether the transport of them is markedly accelerated by insulin or whether any other sugars linked to other substances to form glycosides are also *rapidly taken up*

FISHER I do not know whether Dr Park and I ought to come here as twins and say No together but certainly for myself I have not and it is a very interesting idea

Whilst I am here I would just like to say something about this business of the growth hormone effect *I think a possible explanation is that when* we measured the effect of our Wilhelmi beef growth hormone preparations on the penetration of galactose into the heart we had to have glucose present in the perfusate to slow the penetration down to manageable dimensions and it is quite conceivable that the effect that we obtained there is secondary to the effect on glucose *metabolism*

D ARCY I would like if I may to direct my question to Dr Park I was very interested to hear Dr Park mention that hypophysectomy increases the transport rate whereas growth hormone and cortisone *in vivo* depresses it I would like to know for information if you have tried the effects of corticotrophin either *in vivo* or *in vitro preparations*

PARK We have not tested this at all

D ARCY I have wondered if that may possibly in some way explain the difference between your *in vivo* effects with growth hormone and your opposite results which you found *in vitro* Could it be contamination of your growth-hormone preparation, say with another *trophic hormone* from the pituitary?

PARK Contamination by other factors is always a possibility and insulin-like or hypoglycaemic activity has been observed with pituitary factors other than growth hormone On the other hand an immediate insulin-like activity *in vivo* or *in vitro* has been observed with growth-

PART III

INSULIN AND INTERMEDIARY METABOLISM

Chairman PROFESSOR F G YOUNG

SOME OBSERVATIONS ON THE MODE OF ACTION OF INSULIN

E. B. CHAIN

Istituto Superiore Di Sanità, Rome

The following is a brief summary of some of the work on the mode of action of insulin which has been carried out in this laboratory by the author and his colleagues over the last 8 years.

As is well known Gemill[8,9] made the important observation that insulin stimulates glucose uptake and glycogen synthesis in the isolated rat diaphragm. This observation was confirmed by many authors, among them Villee and Hastings[15] who were the first to use U-^{14}C-labelled glucose and followed the fate of the ^{14}C carbon in various fractions. Their results, summarized in Table I, formed the starting point for the present investigations.

As will be seen from the figures in Table I, while in confirmation of Gemill's results there was an increase in glucose uptake and glycogen synthesis, the larger part of the glucose disappearing from the incubation fluid could not be accounted for, either in the absence or in the presence of insulin, and furthermore the increased synthesis of glycogen in the presence of insulin was insufficient to account for all the 'extra' glucose disappearing from the incubation medium. The author and his colleagues therefore set themselves the task to get a quantitative balance sheet of all the metabolites formed from glucose in the rat diaphragm in the presence and absence of insulin, with the view of ascertaining the fate of the 'extra' glucose metabolized in the presence of insulin.

SEPARATION AND QUANTITATIVE DETERMINATION OF RADIOACTIVE METABOLITES BY PAPER CHROMATOGRAPHY

Over 90 per cent of the radioactive metabolites formed from glucose were recovered in an aqueous alcoholic extract of the homogenized muscle and in the medium of incubation. A small part, shown to be glycogen, remained in the insoluble residue.

The metabolites in the incubation medium and the muscle extracts were separated by paper chromatography and quantitatively deter-

mined by a scanning device In the initial stages of these investigations an apparatus similar to that of Tomarelli and Florey[14] was used in which a paper strip is passed by hand under the window of a Geiger counter and its radioactivity is measured per unit area This was an extremely laborious procedure and can only be used for monodimensional chromatography To achieve a sufficient degree of separation of the metabolites it became necessary to use bidimen

TABLE I

IN VITRO UTILIZATION OF ^{14}C LABELLED GLUCOSE BY RAT DIAPHRAGM MUSCLE

	Normal		Diabetic	
	Without insulin	With insulin	Without insulin	With insulin
Number of animals	14	14	7	7
1 Total glucose disappearing from medium * mg g per hr	1 53 ± 0 046	2 87 ± 0 047	0 78 ± 0 035	2 17 ± 0 10
2 Glucose metabolized to CO_2* mg g per hr	0 11 ± 0 012	0 22 ± 0 026	0 08 ± 0 019	0 11 ± 0 030
3 Glycogen synthesized* mg g per hr	0 17 ± 0 062	0 62 ± 0 13	0 14 ± 0 045	0 61 ± 0.59
4 Glucose unaccounted for mg g per hr	1 25	2 03	0 56	1.45
5 Utilized glucose recovered as CO_2, per cent	7 2	7 7	10 3	5 1
6 Utilized glucose converted to glycogen per cent	11 1	21 6	18 0	28 1
7 CO_2 in centre well derived from glucose in medium per cent	10 4	16 3	8 1	9 2

* The values given are means ± standard error
After Villee and Hastings

sional chromatography and it was therefore essential to devel automatic scanning devices Due to the limited time I shall not e into the details of this work which has been published in full [8] number of models were developed and one now in routine use illustrated in Fig 1 The paper chromatogram is held in a which passes across two end window Geiger counters disc uously from position to position thus exposing for counting a area of a certain size determined by the size of diaphragms ,

CHAIN

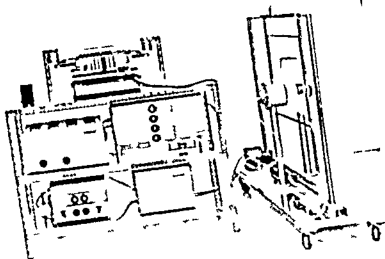


PLATE ~

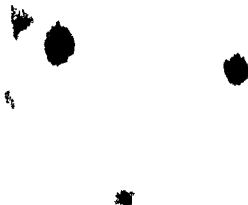


Fig   Number 1   p and cor e pond g rad   aut gram

cross the end windows for a certain time period. The frame is moved first horizontally then when one full line is explored, back o the original position and vertically downwards to explore the next line The time period depends on the intensity of the radiation emanating from the scanned area of the radiochromatogram when here is only background radioactivity the scanning period is 45 seconds if the background threshold is exceeded the paper is exposed five times longer The movements of the paper and the timing operations are controlled electronically there are no gears or lectromechanical parts

The recording device for the Geiger pulses consists of an electric ypewriter the number keys and return key of which are actuated by a series of solenoids An electronic discriminator transmits the number of pulses collected by the Geiger counters per time unit and unit area to the appropriate solenoids on the number keys and writes t on a blank sheet of paper which is moved by the carriage of the ypewriter in synchronization with the radiochromatogram Number maps are thus obtained which give the position of the adioactive spots and their intensity in number of counts A typical number map and its corresponding radiochromatogram are shown n Fig. 2

This model has been further simplified by attaching the Geiger counters directly to the back of the typewriter and using the carriage of the typewriter for moving the radiochromatogram which is stuck with scotch tape to one end of the blank paper sheet inserted n the typewriter carriage (Fig. 3) thus the frame moving the paper chromatogram is eliminated [7]

A simple electronic scanner of monodimensional chromatograms based on a similar principle has also been developed (Fig 4) It has the great advantage over the ratemeter in that it gives the number of counts per unit area scanned from background up to any value A typical monodimensional number map is shown in Fig 5

INFLUENCE OF INSULIN ON GLUCOSE METABOLISM IN THE ISOLATED RAT DIAPHRAGM

The metabolites formed from U [14] C labelled glucose in the extract of rat diaphragm and in the incubation medium are shown in the monodimensional radiochromatograms shown in Fig 6 They have been identified as lactate [1] phosphate esters (in zone g) maltose (m) oligosaccharides and glycogen (gs) A better separation of the

PLATE -

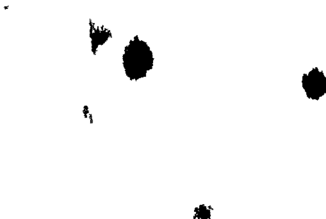


Fig Number m p and or e ponding rad o utog an

across the end windows for a certain time period The frame is moved first horizontally then when one full line is explored, back to the original position and vertically downwards to explore the next line The time period depends on the intensity of the radiation emanating from the scanned area of the radiochromatogram when there is only background radioactivity the scanning period is 45 seconds if the background threshold is exceeded the paper is exposed five times longer The movements of the paper and the timing operations are controlled electronically there are no gears or electromechanical parts

The recording device for the Geiger pulses consists of an electric typewriter the number keys and return key of which are actuated by a series of solenoids An electronic discriminator transmits the number of pulses collected by the Geiger counters per time unit and unit area to the appropriate solenoids on the number keys and writes it on a blank sheet of paper which is moved by the carriage of the typewriter in synchronization with the radiochromatogram Number maps are thus obtained which give the position of the radioactive spots and their intensity in number of counts A typical number map and its corresponding radiochromatogram are shown in Fig 2

This model has been further simplified by attaching the Geiger counters directly to the back of the typewriter and using the carriage of the typewriter for moving the radiochromatogram which is stuck with scotch tape to one end of the blank paper sheet inserted in the typewriter carriage (Fig 3) thus the frame moving the paper-chromatogram is eliminated [7]

A simple electronic scanner of monodimensional chromatograms based on a similar principle has also been developed (Fig 4) It has the great advantage over the ratemeter in that it gives the number of counts per unit area scanned from background up to any value A typical monodimensional number map is shown in Fig 5

INFLUENCE OF INSULIN ON GLUCOSE METABOLISM IN THE ISOLATED RAT DIAPHRAGM

The metabolites formed from U-^{14}C-labelled glucose in the extract of rat diaphragm and in the incubation medium are shown in the monodimensional radiochromatograms shown in Fig 6 They have been identified as lactate [1] phosphate esters (in zone g) maltose (m) oligosaccharides and glycogen (ps) A better separation of the

metabolites is achieved by bidimensional chromatography (Fig 7)

The insulin effect on these metabolites is apparent without any quantitative analysis from a visual inspection of the radiochromatogram given in Fig 8 Glycogen the oligosaccharides and maltose are increased in the presence of insulin while there is no

N
68
61
59
72
57
62
257
815
2148
723
201
54
59
62
54
63
68
59

Fig 5 Part of map of a monodimensional radiochromatogram

apparent effect on the lactic acid phosphate esters or the glucose present in the tissue The results of the quantitative evaluation of the radiochromatograms by the scanning methods outlined above and the determination of radioactive CO_2 formed are shown in Tables II and III They confirm the visual impression and in addition show that insulin has no effect on the production of radioactive CO_2 Since Tables II and III were completed many more experiments using the same technique have been carried out always with the same results

The rapidity of the insulin effect on the polymerization of glucose is shown in Figs 9 and 10 A definite increase in glycogen oligosaccharide and maltose in the presence of insulin is shown already after 3 minutes incubation and is very pronounced at 7 and 15 minutes

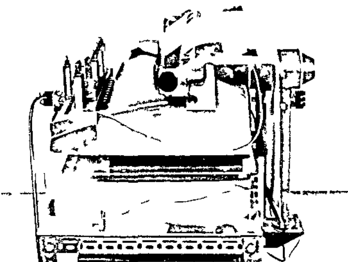
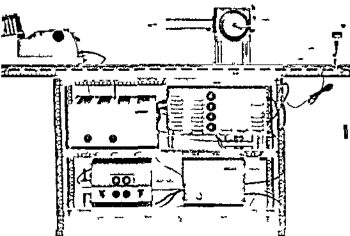


Fig 3 S g d cef r qu tt e b d l d lr g phy



F 4 S d f q tt e d en o l rad ah o atog phy

LATE 4

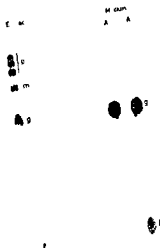


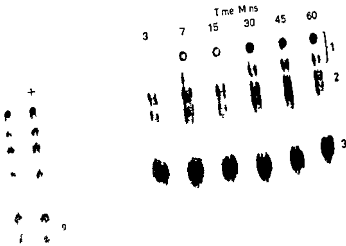
FIG 6 Rad ochron t gram of aqu ous-alc l olic e tra t of r t d pl ragı nuscl a d n ed a before and after incubation witl C-labelled glu

A_1 = M d um before cub t on
A_2 = Mediu after n cubation



7 R d h tog of q co s- l h l e tract of r t d ph gm m scl in d w tl C l belled gl cose
d q l h t g m →b t l th ol NH w t ↓ P cric ac d tert ary b tyl l oh l w ter

PLATE 6



Γ 8 1 d d ph n q u C o n uba ed w h n nly l l u n b u o e nd h u

F G 9 R d h o h of aqu us-a ho e n ub ed f vary ng of a d ph bm b ll d C g u ose period h un o n y

INFLUENCE OF INSULIN ON FRUCTOSE METABOLISM IN THE ISOLATED RAT DIAPHRAGM[2]

Fructose is much less metabolized than glucose by the isolated diaphragm muscle but the same metabolites are formed as are from glucose (Fig 11) Quantitative evaluation of the radiochromato-

TABLE II

INFLUENCE OF INSULIN ON DISTRIBUTION OF ^{14}C IN GLUCOSE METABOLITES IN AN AQUEOUS-ALCOHOLIC EXTRACT OF RAT DIAPHRAGM MUSCLE AFTER INCUBATION WITH UNIFORMLY LABELLED GLUCOSE

Results expressed as counts (on p per) per minut (c p m) × 10 per 100 mg wet wt of diaphragm muscle Insulin 1 unit/ml Gl cose 0 1 p cent 10 μc radio ctive glu ose per Warburg vessel 30-50 mg d aphragm per vessel in 0 6 ml med um ncub t d for 90 m nutes at 37° C in O

Rat	—*ps* Rf 0 003 007						—*m* Rf 0 16		—*g* Rf 0 30 *Glu e + ph phate ester*			
	— *Insul n*			+ *I n l n*			— *Insul*	+ *Insul n*	— *Insul*		+ *Insul n*	
1	29 5			51 8			—	—	26 1		27 2	
2	30 0			59 0			—	—	32 2		32 7	
3	19 1			35 0			6 0	10 2	43 7		47 4	
4a	36 7			52 5			8 8	12 2	44 2		50 7	
4b	20 2			39 4			5 1	9 5	50 0		55 6	
									Glu		*Pl ph te ester*	
									Insul n —	+	*Insul n* —	+
5	11 0			32 6			5 0	8 5	14.5	15 3	7.4	9 7
6	24 5			38 0			6 2	11 6	17 0	18 5	6 5	11 3
	Rf 0		Rf 0 03		Rf 0 07							
	Insul n —	+	*I n l* —	+	*I nl* —	+						
7	11 0	23 1	3 1	7 9	5 6	11 7	5 3	8 7	11 8	15 2	13 6	10.3
8	8 1	33 8	2 2	10 2	4 7	18 8	5 0	24 5	21.2	15 3	4 6	16 0

grams shows that insulin accelerates specifically the same reactions as those influenced in the case of glucose metabolism i e polymerization reactions leading to maltose oligosaccharides glycogen There is again no effect on CO_2 production or lact-

formation nor is there any increase of the amount of free fructose in the tissue

INFLUENCE OF EXTERNAL GLUCOSE CONCENTRATION ON THE PATTERN OF GLUCOSE METABOLISM IN THE ISOLATED RAT DIAPHRAGM IN RELATION TO THE PERMEABILITY THEORY OF THE MODE OF ACTION OF INSULIN

One of the widely current theories on the mode of action of insulin is the permeability theory of Levine and his colleagues[10]

TABLE III

INFLUENCE OF INSULIN ON DISTRIBUTION OF ^{14}C IN GLUCOSE METABOLITES AFTER INCUBATION OF RAT DIAPHRAGM MUSCLE WITH UNIFORMLY LABELLED GLUCOSE

Results expressed as (p m) × 10 [3] per 100 mg wet wt f diaphragm m scle Insulin 1 unit/ml Glucose 0 1 per cent 10 μc radioactive glucose per Warburg vessel 30-50 mg di phragm per vessel in 0 6 ml medium, incubated for 90 minutes at 37 C in O_2

Rat	CO_2		La t a d		Insol ble es due	
	— Insul n	+ I sul n	— I sul n	+ I sul n	— Insul n	+ Insul n
1	45 0	43 0	90 6	102 0	103 6	86 7
2	37 4	42 0	81 5	103 0	71 1	78 8
3	35 8	45 1	37 0	43 0	35 0	48 3
4a	30 4	28 4	48 0	38 4	—	—
4b	25 8	33 5	34 0	42 2	—	—
					Gly ogen insoluble re d e	
					— I s l n	+ Insul 1
5	14 4	12 8	56 8	88 1	2 5	43 5
6	18 0	18 4	34 8	36 6	56 8	14 4
7	24 2	18 0	—	—	8 3	26_
8	36 0	28 4	—	—	11 9	15 1

According to this theory insulin acts by increasing the rate of entry of glucose into the cell If this were the case it should be possible to simulate the specific effect of insulin on glucose metabolism by increasing the glucose concentration Experiments to test this point were made by increasing the glucose in the incubation medium from 0 1 to 2 per cent and quantitatively determining by the scanning method referred to above the free glucose in the tissue and the metabolites formed from it[4] The results are presented in Fig 12

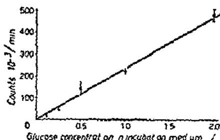


FIG 12 Infl ence of the glu concentration in the m dium on the intracellular con entration of the s l led r t diaphragm

and Table IV It will be seen that the free concentration of in the tissue was proportional to the glucose concentration incubation medium With increasing glucose concentration radioactive CO and the lactic acid increased considerably as pointed out above this does not occur in the presence of The concentration of the oligosaccharides and glycogen also creased up to an external glucose concentration of 0 5 per but above this concentration there was no further effect the gluc polymerizing system was saturated at about 0 3 per cent there was still a very marked specific insulin effect on mal oligosaccharides and glycogen formation in the presence of as h a concentration as 1 per cent glucose as shown in Fig 13 and in the presence of per cent glucose at these high glucose con-

TABLE IV

INFLUENCE OF GLUCOSE CONCENTRATION ON THE DISTRIBUTION OF ^{14}C FROM UNIFORMLY LABELLED GLUCOSE IN RAT DIAPHRAGM MUSCLE

Results expressed c p m × 10^{-3} (counted on p per) per 50 mg b a wet wt. Mean values of 11 experiments ± S E M

Gl n entr t on F act Aq u l h l extract	0 1 per cent	0 25 per cent	0 5 per cent	1 per cent
Zon ps	0 5 ± 3 3	21 0 ± 4 7	41 0 ± 3 7	42 0 ± 10 3
Zone m	3 8 ± 0 6	9 5 ± 1 5	13 5 ± 3 0	17 0 ± 2 6
Zon g	13 6 ± 1 3	46 5 ± 2 5	122 0 ± 32 0	221 0 ± 22 5
Zon l	134 2 ± 10.4	202 0 ± 16 2	256 5 ± 31 2	275 0 ± 28 5
Gly gen f nsol ble resid	8 0 ± 2 3	14 0 ± 2 9	16 5 ± 3 0	17 0 ± 4 4
CO	10 7 ± 8 0	14 0 ± 1 4	15 8 ± 1 7	19 5 ± 2 2

centrations insulin too showed no effect on CO_2 and lactic-acid production The *effect of increasing* the external concentration of glucose on the pattern of glucose metabolism in the isolated rat diaphragm *is therefore qualitatively and quantitatively different* from the specific effect of insulin These experiments are therefore not in agreement with the permeability theory of the mode of action of insulin

The main evidence for the permeability theory of the mode of action of insulin was based on experiments on the influence of insulin on the rate of galactose disappearance from the blood of eviscerated dogs[10] and later on similar experiments by Wick and Drury[16] with eviscerated rabbits and by Park and Johnson[1-] with eviscerated rats These authors found that insulin *accelerated* the rate of disappearance of galactose in the blood and on the assumption that galactose was not metabolized by muscle (the main remaining tissue in eviscerated animals) concluded that this effect was *due to an increase in the permeability of the muscle cells to glucose* The premise on which these experiments were based i e that *galactose is a metabolically inert sugar* was shown to be *incorrect* by Wick and Drury in the eviscerated rabbit and by ourselves as well as Nakada and Wick[11] in the isolated rat diaphragm muscle using radioactive galactose Radioactive CO_2 was found to be formed though in small quantities demonstrating that muscle tissue contains the complete system for metabolizing galactose to CO_2

The experimental findings on eviscerated animals are difficult to interpret in view of the fact that the animals were being continuously infused with glucose in order to counteract the hypoglycaemic effect of hepatectomy and insulin active glycogen and oligosaccharide synthesis was therefore occurring under these conditions The effect of insulin on galactose uptake may therefore be of a secondary nature

It would be more conclusive evidence if an effect of insulin on galactose metabolism could be demonstrated in the isolated rat diaphragm in the absence of glucose Experiments to this end were carried out using radioactive galactose and the quantitative radiochromatographic technique referred to above[4] As shown in Fig 14 the extract of rat diaphragm muscle incubated with galactose contained *only galactose* in measurable amounts small amounts of radioactive CO_2 were also formed Insulin had no effect on the *concentration of free galactose in muscle* diaphragm irrespective

of whether the muscle was incubated for 15 30 60 or
(Table V)

THE UTILIZATION OF GLUCOSE BY RAT DIAPHRAGM MUS
ANAEROBIC CONDITIONS

Another theory of the mode of action of insulin whic
suggested has been that insulin accelerates the hexokinase
generally assumed to be the first step of glucose metab
experiments on the effect of increasing glucose concentratio
pattern of glucose metabolism described in the preced
graph are incompatible not only with the permeability the

TABLE V

EFFECT OF INSULIN ON THE INTRACELLULAR CONCENTRATION OF GALACTOSE-1 IN ISOLATED RAT DIAPHRAGM

Results expressed as c p m × 10 per 50 mg tissue wet wt D incubated in O t 37° in 0 6 ml phosphate-saline medium. Gal. concentration in medium of 0 1 per cent Total radioactivity 1 µc per Warburg vessel approximately 7 × 10 c p m (counted on paper) Number of experiments given in parentheses.

Time of incubation min	Without insulin	With insulin
15	3310 ± 110 (6)	3340 ± 130
30	3420 ± 120 (6)	3370 ± 110
60	3640 ± 560 (4)	3880 ± 230
90	3050 ± 250 (8)	3490 ± 560

also with the hexokinase theory for if the action of insulin on th
pattern of glucose metabolism cannot be simulated by increasing glucose concentrations it follows that it cannot be simulated either by increasing concentrations of the first product of glucose metabolism

It was thought that if insulin acted on the hexokinase system such effect should be demonstrable also under anaerobic conditions after brief incubations in view of the fact that the hexokinase reaction is anaerobic provided sufficient ATP is present To their surprise the present authors found that glucose is practically not metabolized by diaphragm muscle under anaerobic conditions [1,5]
As is apparent from Fig 15 only free glucose is present in
muscle incubated anaerobically with glucose for periods of 3
15 minutes in bicarbonate medium In phosphate buffer to

metabolism of glucose is very reduced under anaerobic conditions, even after 60 minutes incubation very little radioactive lactate was formed (Table VI) However it was noticed that when diaphragm

TABLE VI

FORMATION OF LACTIC ACID FROM GLUCOSE BY RAT DIAPHRAGM MUSCLE INCUBATED UNDER AEROBIC AND ANAEROBIC CONDITIONS

Re ults expressed as c p m $\times 10^{-3}$ of lactic acid *per 50 mg tissue incubated in a* phosphate-buffered medi um conta ning 0 1 per cent gluco.e for 60 minutes Rad oactivity of uniformly labelled ^{14}C glucose 10 μc 7 × 10^5 c p m Mean values given ± s e m Number of experiments g ven in parentheses

I n bated n O_2	*Incubated in* N_2
43 2 ± 4 7 (2)	14 0 ± 1 8 (18)

muscle was incubated first in oxygen in the presence of glucose and then in nitrogen active lactic-acid formation took place but only for a few minutes The presence of glucose was necessary in the

TABLE VII

NFLUENCE OF AEROBIC AND ANAEROBIC CONDITIONS ON LACTIC ACID FORMATION FROM GLUCOSE BY RAT DIAPHRAGM MUSCLE

Results expressed as c p m × 10 $^{-3}$ of lactic acid per 50 mg tissue incubated in a b carbonate-buffered medium containing 0 1 per cent glucose R dio ctivity per vessel 10 μc of uniformly labelled glucose 7 × 10^6 c p m Mean v lue g ven ± S E M Number of experiments g ven in parentheses

Cond t ons	**I cub ated in* O_2 *15 m n*	**I c bated n* N_2 *15 m n*	**Incubated i i O 15 m n N introd ced for 5 m n Inc bated n* N_2 *10 n n*	*I c bat d i O 15 m n N i trod ed for 5 m n *In bated in* N_2 *10 m n*	*†I b ted n O for 15 m* N_2 *int od ced for 5 m n *I cubated in* N_2 *10 m*
Lactic acid	5 3 ± 1 0 (24)	11 7 ± 1.4 (15)	8 0 ± 4 7 (23)	9 5 ± 1 5 (7)	8 0 ± 0 6 (8)

* Incubation in p esence of ^{1} C gluco e
† *Incubation in presence of non radioactive glucose*

aerobic phase radioactive glucose added in the anaerobic phase after previous exposure of the muscle to oxygen in the presence or absence of non-radioactive glucose was converted only in very small amounts to lactic acid (Table VII) *In more recent experiments*

t was found that it was sufficient to expose the rat dia muscle to oxygen or to air for as short a period as 3 minutes presence of radioactive glucose to obtain the spurt of radio actic-acid formation

It seems thus clear that oxygen is an essential requisite for metabolism of glucose and no scheme which does not take of this fact can be considered complete That the failure of diaphragm muscle to metabolize glucose under anaerobic condi s not simply due to a depletion of ATP is apparent from Table V

TABLE VIII

INFLUENCE OF AEROBIC AND ANAEROBIC INCUBATION ON THE ADE OSINETRIPHOSPHATE CONCENTRATION OF RAT DIAPHRAGM MUSCLE

Results expres ed y of ATP per 100 mg d aph gm mu le before in ub t on nd after 3 minut incub ion in a pho ph te nd b ca bonate-buff red medium Mean sults of f fteen experiment ± S E M

I i l	Aft r i cub t			
	B arbo t le m d um		Pl pl te med m	
	Aer b	A e ob	Aerob	An erob
1230 ± 100	560 ± 63	470 ± 31	890 ± 64	630 — 57

which shows that after 7 minutes incubation in nitrogen there is still sufficient ATP present to enable the hexokinase reaction to proceed It should be recalled that under aerobic conditions there is a strong insulin effect on the polymerization of glucose after 7 minutes incubation

The presence of ATP in rat diaphragm muscle after incubation under anaerobic conditions for 7 minutes could also be demonstrated using radioactive phosphate in the incubation medium though naturally its turnover rate was reduced in comparison with that occurring under aerobic conditions (Table IX)

It may altogether become necessary to revise the currently accepted idea that glucose-6-phosphate is the first intermediate from glucose in muscle The experiment reported in Table X is suggestive in this direction[1] In this experiment rat diaphragm muscle was first incubated with ^{14}C-glucose for 30 minutes and then after washing was incubated in the absence of glucose for varying time periods Quantitative radiochromatography of the muscle extracts

TABLE IX

THE INCORPORATION OF ^{32}P INTO ADENOSINETRIPHOSPHATE IN RAT DIAPHRAGM MUSCLE INCUBATED UNDER AEROBIC AND ANAEROBIC CONDITIONS

Results expressed as c.p.m. $\times 10^{-3}$ per 100 mg. tissue referred to an initial activity of the medium of 5×10^{7} c.p.m. (counted on paper). Diaphragm incubated in a phosphate-buffered medium containing 0.1 per cent glucose and 100 μc of $Na_2H^{32}PO_4$. Mean values given ± s.e.m. Number of experiments given in parentheses

Time incubation min	*Adenosinetriphosphate*	
	Aerobic	*Anaerobic*
3	56.0 ± 3.2 (19)	20.3 ± 3.9 (12)
7	58.8 ± 2.3 (17)	24.1 ± 1.9 (8)
15	52.9 ± 3.0 (27)	9.2 ± 1.9 (8)

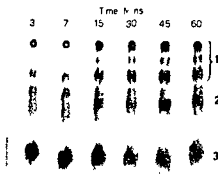
after different times of incubation showed that all radioactive polymerization products of glucose as well as the free glucose in the tissue disappeared rapidly, except the hexose phosphate esters which increased about four times (Table X). One would expect the first

TABLE X

DISTRIBUTION OF ^{14}C AFTER INCUBATION WITH UNIFORMLY LABELLED GLUCOSE FOLLOWED BY INCUBATION IN THE ABSENCE OF SUBSTRATE

Results expressed as c.p.m. $\times 10^{-3}$ per 50 mg. diaphragm muscle. Glucose initially present 0.1 per cent. Activity of medium c.p.m. $- 7 \times 10^{6}$

	Fractions	*After incubation with ^{14}C glucose*	*After incubation in the absence of substrate*		
		30 min	10 min	30 min	60 min
	CO_2	1.2	3.0	5.5	10.2
Aqueous Ethanolic Extract	Zone 1				
	Rf = 0 glycogen	13.7	5.0	4.5	3.6
	Rf = 0.03 oligosaccharide A	4.5	3.2	0.8	0.7
	Rf = 0.07 oligosaccharide B	11.0	3.1	2.9	2.7
	Zone 2				
	Maltose	8.5	2.5	2.5	2.5
	Zone 3				
	Rf = 0.30 glucose	8.8	5.9	2.1	2.7
	Rf = 0.34 hexose phosphates	3.4	11.5	13.4	13.3
	Glycogen of insoluble residue	16.9	7.9	6.5	7.5


3
7
15
30
45
60
1
2
3


4

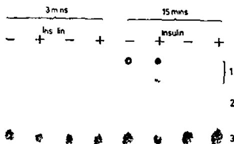
[illegible]



Fɪɢ 11 R d hromat›- gram of q cous-al hol c extract t rat d aphragm muscl me ıb red with un f rmly labelled ¹ C fructose v th nd with t *insul n*

Fɪɢ 13 Rad aut ɢr ph of chromat g n t aque us-ethanoli extract f r t d pl ragn n ı cl ı c bated f r 90 n n tes with ı per c t gluco e w th nd w *thout* ɪ *sul n*

Fɪɢ 14 R d ıutograph of hro natog aı of q cous- th nol c extract f r t di phragm n u cle ıncub ted f r 30 n m tes with o ı per ce t galacto e w th and ıtl ut *n l t*



4

A B A B

[illegible] Rad [illegible] t [illegible] f q [illegible] hol [illegible] f [illegible] d pl [illegible] d with [illegible] glu[illegible]se [illegible] A [illegible] B [illegible]

PLATE 10

CHAIN









Fig 1(R di chr atogra t a ju us- l l l xt ct ot r t di ↓l rag n mu cl ul ated w th Na I O Tv o-d i c i io ial hr mat gra i → P op I a t i io num hydrox i w ter ↓ i butyric c d i i no i t n hydr xide

product of glucose metabolism to be a very reactive substance not to accumulate in an actively metabolizing tissue as the [?] phosphate was shown to do in this experiment

Experiments are in progress with radioactive phosphate with aim to ascertain whether any insulin effect could be shown on principle phosphorylated compounds formed in diaphragm mu[?] The phosphorylated compounds were separated by chromatc[?] (Fig 16) and quantitatively determined by the scanning [?] referred to above No effect of insulin on any of the compou

TABLE XI

THE EFFECT OF INSULIN ON THE INCORPORATION OF ^{32}P INTO ADENOSINETRIPHOSPHATE AN[D] PHOSPHOCREATINE IN RAT DIAPHRAGM MUSCLE INCUBATED IN THE ABSENCE AND PRESENCE OF GLUCOSE

Results exp ssed as c p m. × 10 [?] per 100 mg of tissue incubated for 30 minut s eferred to an initial activity of the medium of 50 × 10[?] c p m. Medium con r ed 100 μc of N [?] ^{32}PO

Experiment	Aden s netr pho phate				Pl p ocreat n			
	N gl e		W'th gluco e		No gl c se		W th gl e	
	N insul n	With insul n	N insul n	W th insul	No is l	W th ul n	No insul n	W th insul n
1	62 1	65 8	62.4	64 4	20 3	13 5	33 8	49 2
2	59 7	66 1	62 7	65 1	13 3	14 0	45 9	47 0
3	73 1	89 1	90 8	112 0	25 8	44 8	59 8	77 7
4	83 6	60 7	75 1	86 3	41 2	50 0	45 7	77 6
5	48 4	68 4	73 0	82 6	17 4	12 7	17 9	55 9
6	46 7	45 7	67 0	51 0	4 4	6 1	29 1	29 6
7	83 6	80 2	68 5	81 9	22 4	26 9	37 5	53 2
8	68 4	63 4	68 9	75 5	16 5	17 1	35 7	39.4
Mean	66 5 ±5 4	67 4 ±4 6	71 1 ±3.2	77 4 ±6 5	20 8 ±3 7	23 8 ±5 0	38 2 ±4 4	48 7 ±7 6

formed could be found except in some experiments but not in all a statistically significant effect on the turnover of phosphocreatine (Table XI) This effect occurred only in the presence of glucose Exp riments to determine the specific activities of phosphocreatine and ATP are in progress

EFFECT OF INSULIN O[N] GLUCOSE METABOLISM IN BROWN ADIPOSE TISSUE[3]

In the brown adipose tissue of the rat glucose is converted [i]n[t]o the same metabolites as it is in the rat diaphragm muscle i.e maltose

oligosaccharides glycogen and lactic acid (Fig 17) In
is actively converted into fat Insulin was shown to
cogen oligosaccharides and *maltose synthesis from g'*
brown adipose tissue of the rat but had no effect on
CO_2 formation In fact it stimulated the same
as it did in the rat diaphragm muscle In addition it
notably the synthesis of fat from glucose (Table XII) T'
brown adipose tissue of the rat insulin could be shown to
two synthetic highly endergonic reactions

TABLE XII

INFLUENCE OF INSULIN ON GLYCOGEN AND FATTY ACID SYNTHESIS FROM ^{14}C GLUCOSE IN RAT BROWN ADIPOSE TISSUE SLICES

Results expressed as c p m × 10^{-2} per mg tissue wet wt Tissue incubated for 90 minutes in 0 6 ml phosphate medium at 37 in O_2 Glucose concentration 0 1 per cent radioactivity 10 μc per vessel 7 × 10^4 c p m (counted on paper) Mean values of eleven experiments given ± s e m

	No insulin	With insulin
Glycogen oligosaccharides and maltose (in aqueous alcoholic extract)	1 2 ± 0 1	3 2 ± 0 5
Fatty acids (in insoluble residue)	9 5 ± 1 5	17 7 ± 1 9

CONCLUSIONS

The authors believe that none of the hitherto suggested hypot
on the mode of action of insulin can account for all the exp
data Insulin appears to exert a specific stimulating effect on the
energy-requiring synthetic reactions occurring in the cells on which
it acts such as glycogen fat and protein synthesis It must act by
making energy available for these reactions probably from a bound
form by a mechanism which up to the present is not understood
and forms the subject of present investigation in the authors
laboratory

REFERENCES

1 BELOFF-CHAIN A CATANZARO R CHAIN E B MASI I POCCHIARI F & ROSSI C (1955) *Proc Roy S c* 143 481
2 BELOFF-CHAIN A CATANZARO R CHAIN E B MASI I & POCCHIARI F (1955) *Bio hem try f Nitrogen* Ed by Suomalainen Tiedeakatemia Academia Scient aru Fennica — Helsinki, 412
3 BELOFF-CHAIN A CATANZARO R CHAIN E B MASI I & POCCHIARI F (1956) *Selected Scient fic P per from the Ist tut S per ore d San tà* 1 345



4 BELOFF-CHAIN A CATANZARO R CHAIN E B MASI I & Po
Sele t d S ient fic P pers f om th I t h to S per e d San tà 1 356
5 BELOFF-CHAIN A CATANZARO R. CHAIN E B LONGINOTTI L
Il th I t C ngr B ochem 6–12
6 CHAIN E B FRANK M POCCHIARI F ROSSI C UGOLINI F & I
Select d S ent fic P per f m the Ist tuto S per e di S n tà 1 239
7 FRANK, M (1959) S l ct d S t fic P pers f n the I t t t S periore d San t.
8 GEMMILL C L (1940) J l ns Hopk H p B ll 66 232
9 GEMMILL C L (1941) J l ns H pk H p B ll 68 329
10 LEVINE, R. GOLDSTEIN M. S HUDDLESTUN B & KLEIN S P (1950) Amer
163 70
11 NAKADA H. I & WICK A N (1956) Amer J Phy i l 185 23
12 PARK, C R. & JOHNSON L H (1955) Amer J Phys l 182 12
13 POCCHIARI F BARONCELLI V BELOFF-CHAIN A CATANZARO R C
LONGINOTTI, L. & MASI I (1958) Il th I t C ngr Bio hem 9
14 TOMARELLI, R M & FLOREY K (1948) Sci e 107 630
15 VILLEE, C A HASTINGS A B (1949) J B ol Ch m 179 673
16 WICK, A N DRURY D R (1953) Amer J Phy i l 173 2 9



INSULIN ACTION AND THE PASTEUR EFFECT IN MUSCLE

P J RANDLE and G HOWARD SMITH

Department of Biochemistry University of Cambridge

The uptake of glucose by many cells and tissues is known to be greater under anaerobic than under aerobic conditions This stimulation of glucose metabolism in the absence of oxygen first recognized by Pasteur is at present thought to depend upon the fact that anaerobic metabolism of glucose yields a smaller amount of energy-rich phosphate than aerobic metabolism In conformity with this view substances such as 2 4-dinitrophenol which inhibit the production of energy rich phosphate during respiration increase the uptake of glucose by cells and tissues under aerobic conditions

Insulin also increases the aerobic uptake of glucose by certain mammalian cells and tissues such as skeletal and cardiac muscle adipose tissue leucocytes fibroblasts and lens The mechanism of this effect of insulin on glucose uptake is not yet known though in the case of skeletal and cardiac muscle it is thought that the overall rate of uptake of glucose is limited by its rate of entry into the muscle cell and that insulin acts by accelerating this process (Levine and Goldstein[3] Park and Johnson[6] Park *et al*[7])

This idea necessarily implies that any other factor which increases the uptake of glucose by muscle must also accelerate the entry of glucose into the cell It was therefore important to know whether anoxia or substances which inhibit oxidative phosphorylation would increase the uptake of glucose by muscle in a similar manner to insulin i e by accelerating the entry of glucose into the muscle cell We present here a comparison of the effects of insulin with those of anoxia and of substances which inhibit oxidative phosphorylation on the uptake of glucose and the intracellular accumulation of glucose and xylose by isolated rat diaphragm A detailed account of most of the experimental methods used has been published elsewhere (Randle and Smith[8 9])

UPTAKE OF GLUCOSE BY ISOLATED RAT DIAPHRAGM

The uptake of glucose by isolated rat hemidiaphragm incubated

F

in a medium buffered with bicarbonate (measured as the change in glucose concentration in the medium during incubation for 1 hour at 37 C) was found to be increased by anoxia and by 2 4-dinitrophenol (0 05 mM or 0 25 mM) sodium arsenite (mM) sodium cyanide (mM) and sodium salicylate (5 mM) as well as by insulin (0 1 unit/ml) The uptakes ranged from about 150 per cent of the basal uptake in the presence of salicylate to about 400 per cent when cyanide or insulin were present (Fig 1) On the other hand when diaphragm was incubated in a medium buffered with phosphate

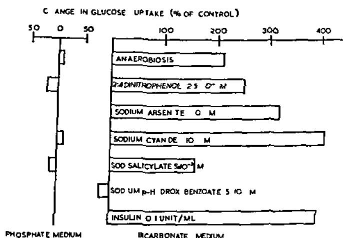


FIG 1 The effect of insulin anaerobiosis or some cell poisons on the uptake of glucose by isolated rat hemidaphragm incubated in media buffered with phosphate or bicarbonate

glucose uptake was not increased by anoxia 2 4-dinitrophenol sodium cyanide or sodium salicylate (Fig 1) in conformity with the results of others who have used phosphate-buffered media (*Demis* and Rothstein[1] Smith and Jeffery[10] Villee *et al*[12] Walaas and Walaas[13]) The lack of effect of these factors on glucose uptake in phosphate medium appears to be due to inadequate buffering under these conditions (Randle and Smith[8]) We conclude therefore that the uptake of glucose by isolated diaphragm like that of other cells and tissues is increased by anoxia or by substances which inhibit oxidative phosphorylation provided adequately buffered media are used

In order to determine whether anoxia and substances which inhibit oxidative phosphorylation might increase uptake of glucose by diaphragm by accelerating its entry into the muscle cell we have also investigated the effect of these factors on the accumulation of free D-glucose and D-xylose in isolated diaphragm.

ACCUMULATION OF GLUCOSE AND XYLOSE IN ISOLATED RAT DIAPHRAGM

The amount of a metabolizable sugar such as glucose which is present in the free form within a cell must depend upon the relative rates of its entry into the cell and of its further metabolism (see Fig 2) In

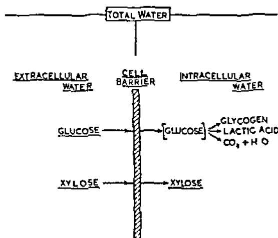


Fig 2 Factors involved in the intracellular accumulation of f e gl cose and xylose in r t d phragm.

diaphragm under normal conditions the latter exceeds the former and no free glucose accumulates (Park and Johnson[6]) although with variation of conditions the rate of entry may be enhanced or the rate of utilization depressed to such a point that the order is reversed and free glucose becomes detectable (Park *et al*[7]) On the other hand this difficulty does not arise if a sugar such as xylose is used D-xylose does not appear to be metabolized by diaphragm to any appreciable extent (Kipnis and Cori[7]) so that the rate at which it accumulates within the muscle cell may be expected to depend solely upon its rate of entry Accordingly we have examined the

effects of insulin and of factors which inhibit oxidative phosphorylation on the intracellular accumulation of glucose and xylose in isolated rat diaphragm

In what follows the amount of free sugar which accumulates in diaphragm is expressed as a space the glucose or xylose space being that fraction of the volume of the tissue which contains fluid of the same specific gravity and glucose or xylose concentration as the incubation medium Interpretation of these results in terms of the distribution of sugar between extracellular and intracellular fluids involves comparing the glucose or xylose space with the volume of extracellular fluid Glucose or xylose is deemed to be present in intracellular water only if the glucose or xylose space exceeds the volume of extracellular water The volume of extracellular water has been determined by measuring the space occupied by inulin raffinose sucrose or thiosulphate substances which are thought to be confined to extracellular fluid under normal conditions We have found as others have that not all these substances yield the same value for the volume of extracellular fluid the smallest value being obtained with inulin and the greatest with thiosulphate Since it is not known which substance yields the most reliable estimate of extracellular fluid volume the conclusions which we draw are for the most part based on all estimates of the volume of extracellular fluid

Spaces were measured in an intact diaphragm preparation (*Kipnis* and Cori[2]) in which none of the muscle fibres are cut The advantage of this preparation is that its spaces are apparently similar to those of the muscle *in vivo* Intact diaphragms were *incubated singly* in bicarbonate medium usually for 1 hour and the medium and tissue then analysed and the spaces calculated

The distribution of free glucose between extracellular and intracellular water in isolated diaphragm under various conditions is shown in Fig 3 It will be observed that when diaphragm was incubated in bicarbonate-buffered medium in the presence of oxygen free glucose appeared to be confined to extracellular fluid This was still the situation when insulin arsenite cyanide or salicylate were present or oxygen excluded even though the uptake of glucose was greater in every instance (cf Fig 1) This must mean either that the potential rate of intracellular utilization of glucose under these conditions exceeds its rate of entry such that no free sugar accumulates or that glucose does not enter the muscle cell as the free sugar

When diaphragm was incubated in the presence of 2 4-dinitrophenol however not only did the uptake of glucose increase (Fig 1) but some free glucose accumulated in intracellular water (Fig 3) Furthermore when sodium fluoride (which inhibits utilization of glucose) was present in addition to dinitrophenol even more glucose

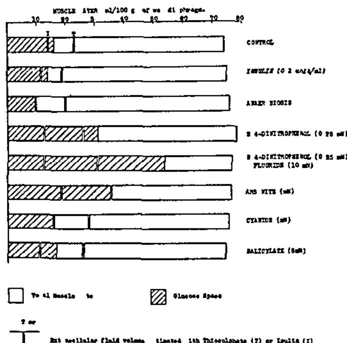


FIG 3 The distribution of free glucose between extracellular and intracellular water in intact rat diaphragm incubated in bicarbonate medium In each instance the breaks in the histogram are on the left the limit of the inulin space on the right the limit of the thiosulphate space

accumulated intracellularly Hence unless the process of glucose uptake is abnormal when dinitrophenol and fluoride are present it must be concluded that glucose does indeed enter the muscle cell as the free sugar and that it accumulates only when the rate of utilization within the cell falls below the rate of entry These results are therefore compatible with the view that it is the entry of

glucose into the muscle cell which is the rate-limiting step in glucose uptake and that insulin increases glucose uptake by accelerating the entry of glucose Furthermore it would appear that anoxia and substances which inhibit oxidative phosphorylation likewise in crease glucose uptake by accelerating its entry into the muscle cell

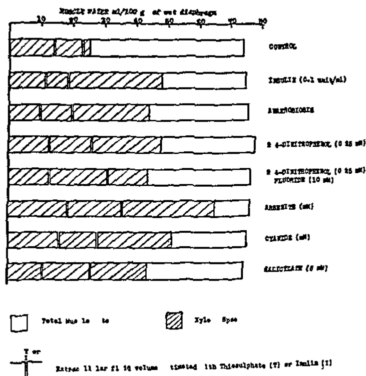


Fig 4 The distribution of free xylose between extracellular and intracellul r water in intact rat di phragm incubated in bicarb nate medium In e ch instan e the breaks in the hi togram a e on the left the limit of the inulin space on the right the limit of the thiosulphate sp ce

Further support for this view is provided by the results of a similar series of experiments with D-xylose (Fig 4) Under basal conditions a small amount of xylose accumulated in intracellular water (equivalent to equilibration with about 6-20 per cent of intra cellular water) but in the presence of insulin arsenite cyanide 2 4-dinitrophenol (with or without fluoride) or salicylate or in the

absence of oxygen the accumulation of xylose was greatly enhanced (equivalent now to equilibration with 40–60 per cent of intracellular water) We conclude that each of these factors also accelerates the rate of entry of xylose into the muscle cell Although we do not yet know whether glucose enters the muscle cell by the same or a similar process to xylose it none the less seems reasonable to infer that these results lend support to the view that anoxia and substances which inhibit oxidative phosphorylation increase glucose uptake by accelerating the entry of the sugar into the muscle cell

SPECIFICITY OF EFFECTS OF ANOXIA AND CELL POISONS ON PERMEABILITY

The investigations of Levine and his associates (see Levine and Goldstein[3]) and of Park and his colleagues suggest that the effect of insulin on the penetration of muscle cells by sugars has elements of specificity For example sorbitol and mannitol although possessing physicochemical properties similar to those of glucose and other sugars do not enter the muscle cell either under basal conditions or in the presence of insulin. It is therefore pertinent to consider whether factors which inhibit oxidative phosphorylation accelerate the entry of sugars into the cells of diaphragm in a specific manner or whether they damage the cell barrier* in such a way that substances leak through in a non-specific manner We believe that the process is a specific one for the following reasons

1 The rates of uptake of glucose and of xylose are always very different The extra uptakes of glucose (mg of glucose/g of wet diaphragm/hr) ranged from 1 5 when salicylate was present to 6 4 with cyanide whereas the corresponding figures for xylose (mg of xylose/g of wet diaphragm/hr calculated directly from the xylose content of the tissue) were 0 45 and 0 55 If the accelerated entry of glucose and xylose was due to a breach in the cell barrier then we would expect the two sugars to enter the muscle at more comparable rates Furthermore we would expect free glucose to accumulate within the muscle cell under these conditions

2 With each of these factors the muscle cell still exhibits selective permeability Thus in each case insulin anoxia and cell poisons

* The view that the entry of glucose into the cell is the rate-limiting process in its metabolism would seem to imply that the cell is surrounded by a limiting structure distinct from the major portion of the cell. While there is a considerable body of evidence for the existence of such a structure a consideration of this is not relevant to our present discussion and in order to avoid as far as possible any preconception as to its nature we will refer to it as the cell barrier

promoted uptake of glucose and accumulation of xylose (Figs 1 and 3 and Table I) and also of fructose and ribose (unpublished observations) but not of inulin maltose raffinose sucrose or thiosulphate (Table I) (The very small changes in the inulin maltose raffinose sucrose and thiosulphate spaces observed may be reasonably attributed to alterations in the volume of extracellular fluid) On the other hand if diaphragm was injured by cutting or by freezing with solid carbon dioxide extensive accumulation of inulin raffinose or thiosulphate as well as of glucose or xylose occurred (unpublished observations)

TABLE I

EFFECT OF INSULIN ANOXIA OR CELL POISONS ON SPACES OF RAT DIAPHRAGM

	Changes in spaces (µl /100 mg of wet muscle)					
	Inulin	*Maltose*	*Raffinose*	*Sucrose*	*Thiosulphate*	*Xylose*
Control	(14)	(16)	(20)	(21)	(23)	(26)
Insulin	−2	2	−4	0	−4	+2
Anaerobiosis	−4				−3	+18
2 4 Dinitrophenol	−1	+6	+6	+4	+3	+2
Cyanide	+5				+5	+6
Salicylate	−3	−1	0	−1	+3	+18

3 The enhanced rate of glucose uptake under anaerobic conditions was immediately reduced and the concurrent loss of potassium arrested by restoration of aerobic conditions during the course of the incubation (Fig 5) If the cell barrier had been irreversibly damaged during the anaerobic phase of the experiment it is to b expected that the loss of potassium and the more rapid uptake of glucose would have continued despite the readmission of oxygen

4 It was observed that insulin was still capable of increasing glucose uptake in the presence of 2 4-dinitrophenol arsenite cyanide or salicylate or in the absence of oxygen (Fig 6) As integrity of cell structure is believed to be essential for an effect of insulin it is not likely that it would have exerted an effect under anaerobic conditions or in the presence of these cell poisons if the cell barrier had been damaged by these agents

We conclude on the basis of these observations that factors which inhibit oxidative phosphorylation do not promote the entry of

sugars into the muscle cell by damaging the cell barrier Our results suggest rather that like insulin they accelerate the entry of sugars by affecting the activity of a process which shows some degree of specificity

REGULATION OF SUGAR ENTRY AND *MECHANISM OF INSULIN ACTION*

Although the details of the process whereby glucose enters the muscle cell are still obscure experimental evidence appears increasingly to support the view that a transport process is involved *This may be envisaged as occurring in some manner such as that shown*

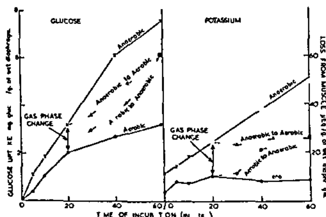


FIG 5 The effect of anaerob o s on the upt ke of glucose and loss of potassium by solated rat hemidiaphr gm incub ted in b carbonat med um

in diagrammatic form in Fig 7 Glucose is *depicted as combining* with a specific constituent of the cell barrier at its outer surface Thence it is conveyed in combination with such a carrier to the inner surface of the cell barrier there to be released into the intracellular milieu Since a number of factors which inhibit oxidative phosphorylation appear to be capable of accelerating the entry of glucose into the muscle cell it seems reasonable to deduce that the activity of the putative transport system is in some way inhibited by energy-rich phosphate One way in which this could occur is shown in Fig 7 We suggest that the putative carrier may occur in

two interconvertible forms differing perhaps by a phosphate residue the phosphorylation occurring by reaction with an energy rich phosphate compound The energy-rich phosphate compound is depicted here as adenosine triphosphate (ATP) but it does not have to be this particular energy-rich phosphate compound If as we suggest only the non-phosphorylated form of the carrier is capable

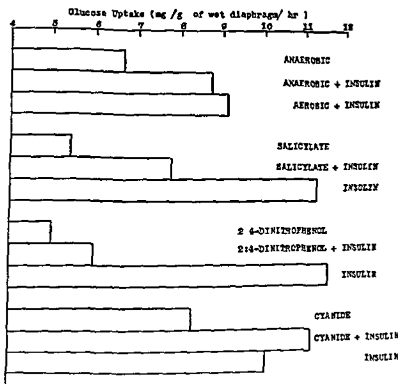


FIG 6 Influence of anaerobiosis or cell poisons on the response to insulin of isolated rat hemidiaphra m incubated in bicarbo ate medium

of combining with the sugar and of transporting it into the cell then our results would receive some explanation Thus a deficiency of ATP induced for example by anaerobiosis (Ottaway[5]) would favour the non-phosphorylated form of the carrier and the transport of sugar into the cell

Since insulin does not inhibit oxidative phosphorylation (Stadie[11])

the mechanism of its effect on sugar transport is likely to be different from that suggested for anaerobiosis and substances which do inhibit oxidative phosphorylation One possibility depicted in Fig 7 is that insulin may increase the proportion of free carrier (and thereby accelerate transport of sugars) by interfering specifically with phosphorylation of the carrier in the cell barrier Thus it might do either by inhibiting phosphorylation of the carrier or by stimulating dephosphorylation of the phosphorylated carrier

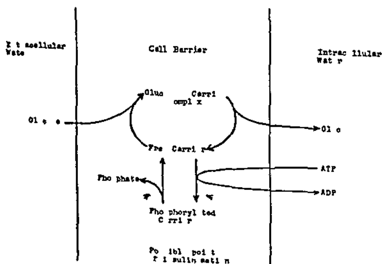


FIG 7 An hypothesis fo the me ha ism of ction of ins l n

This hypothesis of the regulation of entry of sugars into the muscle cell and of the mechanism of action of insulin is patently tentative Alternative interpretations of the experimental observations are undoubtedly conceivable at the present time Nevertheless the hypothesis outlined above does appear to have certain merits and advantages from the biological standpoint It implies for example that the rate of entry of glucose into the muscle cell is geared directly to the energy requirements of the cell It is also capable of providing some explanation for the increased uptake of glucose by skeletal muscle during contraction for in contracting muscle much of the available ATP may be bound to the myofibril

and so not available to the process which we suggest is restricting the entry of glucose into the cell in the resting state Finally it is of interest to note that the mechanism of action of insulin which we put forward has certain elements in common with those proposed on the one hand for the thyroid hormone which is believed to interfere with oxidative phosphorylation and on the other hand for glucagon which is thought to promote the formation of the active (in this case phosphorylated) form of liver phosphorylase

The particular significance of the effects of sodium salicylate on glucose uptake by isolated diaphragm in relation to the use of salicylates in the treatment of diabetes has been discussed elsewhere (Manchester *et al*[4])

REFERENCES

1 DEMIS D J & ROTHSTEIN A (1954) *Amer J Physiol* 178 82
2 KIPNIS D M & CORI C F (1957) *J biol Chem* 224 681
3 LEVINE, R & GOLDSTEIN M S (1955) *Recent Progr Hormone Res* 11 343
4 MANCHESTER K. L RANDLE P J & SMITH G H (1958) *Brit med J* 1 10 8
5 OTTAWAY J H (1955) *Biochem J* 61 441
6 PARK C R & JOHNSON L H (1955) *Amer J Physiol* 182 17
7 PARK C R BORNSTEIN J & POST R. L (1955) *Amer J Physiol* 182 12
8 RANDLE P J & SMITH G H (1958) *Biochem J* 70 490
9 RANDLE, P J & SMITH G H (1958b) *Biochem J* 70 501
10 SMITH M J H & JEFFREY S W (1956) *Biochem J* 63 524
11 STADIE W C (1954) *Physiol Rev* 34 52
12 VILLEE C A DEANE H W & HASTINGS A B (1949) *J cell comp Physiol* 34 159
13 WALAAS E & WALAAS O J (1952) *J biol Chem* 195 367

DISCUSSION

YOUNG Many investigators have perhaps thought of the possibility of designing a machine which would not only make measurements but which would also write up the results Professor Chain and his colleagues have got nearer to this realization than most people Certainly those of us who have had the privilege of seeing the results of his labours find them most impressive the machines he uses are indeed astonishing

LEVINE I was very much interested and intrigued by both of these papers I should like to discuss them separately if I may

Professor Chain in his previous publications and again now has drawn attention to oligosaccharides in muscle presumably intermediates between glucose and glycogen These observations are in accord with the finding by Dr Fishman in Boston of maltotriose and maltotetraose in liver

Now in relation to the action of insulin directly Professor Chain makes the point that mere penetration or availability of glucose to the interior of the cell cannot explain the action of insulin since a high glucose level leads to an increase not only in oligosaccharides but also in the CO_2

and lactic acid while if insulin is added he does not find an increase in the lactic acid or CO Thus finding differs however from the results of many other workers so that we must note that many workers have found using labelled glucose that insulin does increase the yield of CO_2 and I will refer to the latest of these from Dr Renold and his group Using the isolated fat pad the addition of insulin with uniformly marked glucose shows an increase in the yield of CO and I believe Professor de Duve found similar results in other tissues (See also Villee using diaphragm *J Biol Chem* 1949)

The finding of an increased turnover of creatine phosphate in relation to insulin action is of interest as a datum but I should like to know from Professor Chain how he correlates this with the intense stimulation of glucose uptake glycogen formation and fatty acid synthesis in adipose tissue which as far as I am aware does not contain creatine phosphate at all In other words fat tissue which is very insulin sensitive does not contain the creatine phosphate mechanism and if that is supposedly the primary point of insulin action it fails to explain insulin action on fat tissues

Coming now to Dr Smith s presentation of Dr Randle s paper it indicates that substances other than insulin may stimulate the transport system in the diaphragm or in other tissues for that matter Some years ago we had shown that muscular exercise does so presumably by humoral mechanism and now it is shown that anaerobiosis and certain drugs increase the rate of a transport system which according to the studies here reported is specific for certain sugars of particular structure

How are we to relate all these findings? With the permission of the Chairman who asked us to do so I should like to let my hair down It will therefore be a mixture of fact and fancy I hope not too much fancy I believe that the effect of insulin in stimulating in some manner the transport of glucose and other stereospecific sugars into certain cells has been amply demonstrated I would agree with Professor Cori s remark that this does not mean that we know the mechanism of action We are perhaps nearer to it but we still do not know the mechanism of action of insulin The fact that many substances and conditions other than insulin may be shown also to have an effect on the transport system we should like to explain in the following manner The transport system is probably present in all cells metabolizing sugar yet some cells do not show sensitivity to the action of insulin We believe therefore that insulin probably does not act directly on the transport system itself However certain cell systems such as muscle the connective tissues and the specialized connective tissues like fat cells may have this transport system shielded under most conditions in the absence of insulin This explains the fact that in a fasting animal where the total output of sugar

by the liver is small where about two-thirds of that output is utilized by brain that the animal does not become acutely hypoglycaemic Most of the metabolic mass which can utilize sugar like muscle and fat tissue is closed off *so to speak or its transport* gates are firmly locked However these locks can be removed hormonally by insulin under artificial conditions by certain agents like dinitrophenol or anaerobiosis and by physiological conditions like muscular work This therefore means that we have to look for something which ordinarily inhibits transport *in the insulin-sensitive* tissues and that insulin removes this inhibition

Perhaps the findings of Dr Park that under the influence of anterior pituitary hormone transport is inhibited may lead to the elucidation of the nature of the cover for the transport system itself

Now as to the differences between glucose at high concentration which *should give the same results* as does insulin but under certain conditions does not I believe in the first place that one can cull from the literature many individual experiments in which perhaps all of the actions of insulin have been imitated by high sugar administration perhaps not from the same laboratory not at the same tissue but as a general picture The intracellular enzymatic systems in the tissues are *controlled by factors* other than hormones Let us for example take CO_2 production The rate of the Krebs cycle for example is controlled by factors other than insulin and may in a diaphragm taken from a normal animal be already at a height which cannot be stimulated by additional glucose entry In other words the conditions are set in the *cell If one takes the diaphragm* from an alloxanized animal the amount of isotopic CO_2 production from glucose is certainly less than in the normal animal At the present moment in spite of the very interesting experiments of Professor Chain it would be too premature to give up the notion that the most *important, if* not the sole action of insulin on *peripheral tissues including* muscle heart and fat tissue is to remove an inhibition to the normal rate of transport of sugars into the cell interior and that the state of the interior then determines the fate of the sugar

YOUNG Would you like to comment briefly Professor Chain?

CHAIN Let us first come to *the rather essential point whether insulin* does or does not increase CO production We are certain that it does *not* in the rat diaphragm under conditions in *which there is* a marked insulin effect on the polymerization of glucose and the same applies to the brown adipose tissue I should perhaps mention that as Dr Levine *has pointed out some authors have reported a stimulating effect* of insulin on *CO production in the isolated rat diaphragm* and for this reason we have carried out a very large number of *experiments* to see whether or not we could confirm such effect Though in a few *experiments we noticed an apparent effect* this was not found to be statistically significant

but simply due to biological variations It is axiomatic, but not always remembered by all investigators that no cogent conclusions can be drawn in this field of research unless a sufficiently large number of experiments to allow statistical analysis is carried out It is also certain from our experiments that insulin does not produce an increase in lactic-acid formation from glucose while increasing the external glucose concentration in the incubation medium markedly stimulates both CO_2 production and lactic acid production

Finally it is not possible to increase the formation of fat by increasing the external glucose concentration in the white adipose tissue above a threshold value of about 0 3 per cent in the spectacular way insulin does in fact it would be difficult to understand thermodynamically how a series of reactions as endergonic as fat synthesis could be stimulated by changes of concentration of a substrate like glucose within physiological range which is removed by many reaction stages from the end product

In my own view these facts show irrefutably that the mode of action of insulin cannot be explained by a theory like the permeability theory which postulates that insulin acts by increasing the intracellular concentration of glucose

We are far from offering a detailed alternative explanation of the mode of action of insulin all we can say at present is that insulin enables the tissue cells on which it acts to carry out a larger amount of energy requiring synthetic reactions (polysaccharides fat and protein synthesis) that it can accomplish in its absence Several mechanisms by which this could be done can be visualized and as has been already mentioned this is one of the main problems which form the object of our present research

I should like some member of this audience of distinguished specialists in the field of intermediate carbohydrate metabolism to offer some explanation of the results of our anaerobic experiments *There is no question* that without oxygen there is no glucose metabolism Why is this so? Some factor which no one seems to have discussed yet is evidently missing which brings oxygen into the scheme of glucose metabolism and glycogen synthesis We do not know the nature of this factor but it is certainly not ATP and I feel that without knowledge of this factor it will be difficult to understand the mode of action of insulin

KRAHL Leloir has shown that glycogen can be formed by liver from uridine diphosphoglucose The possibility has occurred to me that there may be an insulin stimulation of a pathway from glucose to glycogen via uridine diphosphoglucose or other analogous nucleotides I should like to ask Professor Chain whether with his techniques he could identify such intermediates in muscle and measure their concentration and radioactivity in relation to insulin effects

CHAIN It is interesting that you should have mentioned this This is

of course a field in which we are actively engaged at the present. We also believe that something of that sort is involved in the transport of glucose and glucose utilization

We have got some radioactive nucleotides out of the rat diaphragm, and we have got them particularly out of liver but we are just in the process of identifying them and I would not like to say too much about them except that one of the liver nucleotides which we have got shows a high specific activity indicating a considerable turnover But this is a problem under active investigation

CORI I should like to mention that these uridine necleotides are formed by way of glucose 1 phosphate At the present time there is no other mechanism known for their formation.

Secondly the Leloir enzyme is present in low concentration both in liver and muscle about one-hundreth the concentration of phosphorylase Now while the enzyme exists it does not necessarily follow that it functions If it could function in the direction of synthesis phosphorylase could have its main function as a glycogenolytic enzyme This is a possible mechanism

CHAIN May I ask Professor Cori it is true that at present there is no pathway known apart from the classical one but does that necessarily mean that no other pathway exists

CORI I didn t say that no other pathway exists I merely said that in order to form uridine diphosphoglucose you have to go through glucose 1 phosphate Now the glucose 1-phosphate could be utilized directly by the phosphorylase or it could go by another enzymatic step to uridine diphosphoglucose and then be used by the Leloir enzyme but this enzyme is not very active The question is whether the activity of this enzyme is of sufficient magnitude to explain the rate of glycogen formation in liver and muscle

LEVINE Professor Chain contrasts the seeming complexity of the transport theory with his simple concept Insulin somehow raises the energetic level Such a statement is far from simple No theory proposed for the action of insulin has yet reached the molecular level I do believe however that there is a certain amount of evidence for the facilitation of penetration especially when one considers that such materials as galactose xylose arabinose and others which are not phosphorylated to any extent in peripheral tissues are aided in their penetration into various cells by insulin

BESSMAN Professor Chain s mention of our results prompts me to present them together with our reasons for performing such experiments

We tested the possibility that insulin might act on carbohydrate metabolism in tissue essentially deprived of a membrane by cutting rat diaphragm into pieces so small (1-3 millimetres square) that d xylose

could penetrate them to the same extent without insulin as it could penetrate the intact diaphragm with insulin The addition of insulin to such pieces does not enhance the penetration of xylose significantly When such pieces are included in a medium containing both glucose and xylose there is the same penetration of xylose with or without insulin but glycogen synthesis occurs only in the presence of insulin Since there is apparently good permeability in this preparation without insulin and yet there is no glycogen synthesis when such penetration has occurred we interpret these data to mean that the *role* of insulin in stimulating glycogen synthesis is not *primarily* the effect of insulin upon cell permeability Insulin obviously acts on the cell to improve its permeability but this is the result of a direct effect upon carbohydrate utilization as our experiments with damaged cells demonstrate

This brings me to the reason for such an experiment The preoccupation with membrane transport as the site of action of insulin has led to a general neglect of what appears to be a more specific site of action of insulin namely the energy generating system Professor Chain has pointed out a number of metabolic peculiarities resulting from insulin action which cannot be explained on the basis of a *direct* effect of insulin on transport of substrate across cell membranes I should like to point out certain other highly improbable assumptions which must be made to support a transport theory of the action of insulin The effect of insulin on transport now embraces a large and varied group of compounds including sugars natural and unnatural amino acids of every variety and potassium ions The likelihood that all of these substances are moved into the cell by the same mechanism is very small indeed Secondly the effect of toxic substances such as cyanide and dinitrophenol on facilitating membrane permeability suggests that energy metabolism is the question not specific transport mechanisms The effect of exercise on stimulating carbohydrate uptake without insulin also calls for supplementary assumptions and finally the relation of different tissues all of which utilize glucose to the syndrome of insulin deficiency is not explained by a permeability hypothesis I mean by this that brain which uses glucose almost exclusively does not require insulin at all that diabetic muscle has a normal ability to utilize all substrates except glucose and that diabetic liver apparently can utilize no substrate normally

For these reasons among others we have attempted to develop a unified hypothesis for the site of action of insulin We believe that insulin connects mechanically the enzyme hexokinase to the mitochondrion This function of insulin as a biochemical organizer would produce all of the physiological effects which have been ascribed to insulin

It is well known that mitochondria oxidize inefficiently in the absence of an acceptor of ATP which can return ADP to the site of oxidative

phosphorylation The attachment of hexokinase to the mitochondria provides such an acceptor Brain mitochondria in contradistinction to the mitochondria of the rest of the body have hexokinase attached firmly This obviates the need for the proposed function of insulin in brain

Muscle has an excellent acceptor in the form of the creatine kinase-creatine system and requires no additional acceptor for normal oxida tion In fact exercise which stimulates turnover in the creatine kinase system stimulates diabetic muscle to take up glucose

The membrane transport mechanisms and I emphasize the plural, all *require energy which must be furnished by an efficiently functioning* mitochondrial system From such a system comes the energy for protein synthesis acetylation phosphorylation of thiamine and the many other anabolic functions which are deficient in the diabetic state

In order to accept the transport theory of insulin action we are obliged to say that the deranged metabolism of the diabetic liver is completely secondary to the minor change in muscle metabolism caused by insulin lack From the acceptor-insulin hypothesis however we can see that the abnormal metabolism of the liver results from the lack of an adequate acceptor system equivalent to muscle The liver *requires* the hexokinase system to be attached to the mitochondria for there is very little creatine *kinase activity in liver therefore the liver is more dependent* on insulin for energy metabolism than muscle

Thus we have an opportunity to unify the physiological picture of the requirement of different organs for insulin with a single site and mechan ism of action of insulin the connection of hexokinase to mitochondria to provide an acceptor reaction for energy generation (A *Contribution* to the Mechanism of Diabetes Mellitus p 133 in *Fat Metabolism* edited by Victor A Najjar The Johns Hopkins Press Baltimore 1954)

PART IV

INSULIN AND THE LIVER

Chairman PROFESSOR C F CORI

INSULIN AND GLYCOGEN SYNTHESIS IN THE LIVER

CHRISTIAN DE DUVE

Laboratory of Physiological Chemistry Lou . n

The problem of the action of insulin on glycogen synthesis in the liver has been dealt with before in publications from Professor Bouckaert s laboratory and from our own (Berthet *et al* [1] Bouckaert and de Duve [2] de Duve [3] and Hers and Bouckaert[4]) but remains a chronic one The most recent attempt to solve it has been made by Levin and Weinhouse [6] These authors have made a careful time-course study over periods ranging between 5 minutes and 4 hours of the incorporation of radioactivity in liver and muscle glycogen and in blood CO on rats injected with a trace dose of uniformly labelled ^{14}C glucose with or without insulin (.0 U/kg) Three groups of animals were used rats fasted .0 to .4 hours with a very low initial level of hepatic glycogen similar animals receiving 0 9 g of unlabelled glucose by stomach tube at the beginning of the experiment and post absorptive rats having a liver glycogen content of approximately . per cent The administration of . inhibited the net synthesis of liver glycogen in the first two . and caused glycogenolysis in the third it prevented almost . pletely the incorporation of labelled glucose into hepatic glyco. in all three groups while stimulating the appearance of ^{14}C muscle glycogen and blood CO Hypoglycaemia occurred in animals even in those receiving glucose The authors sum up results as follows Under the conditions employed here none of the glucose which disappeared quickly from the blo the influence of insulin entered the liver glycogen despite the marked effect in muscle

There can of course be no quarrel with this statement but one may question whether the conditions employed appropriate for the study of the physiological effects of

The authors use glucagon free insulin and therefore ap. avoid one of the dangers which we have denounced before ever they furnish no evidence that the insulin they have fact entirely devoid of glucagon In our experience some

purported to be glucagon-free and causing no appreciable initial hyperglycaemia when injected intravenously into the intact animal, still contain measurable amounts of glucagon, as assayed by the more sensitive liver-slice or phosphorylase methods Such amounts could inhibit glycogen synthesis during the early part of treatment but it is doubtful that their effect could extend over the full experimental period of 2 to 4 hours

Much more damaging to the validity of the experiments is the fact that practically all the animals were hypoglycaemic at the time of sacrifice The strong dependence of the glucose-glycogen balance of the liver on the blood-sugar level has been repeatedly emphasized In the present case the authors rule out a disturbing influence of hypoglycaemia on the grounds that insulin failed to stimulate the incorporation of radioactive glucose into liver glycogen even in the post-absorptive animals with high glycogen stores

However this reasoning would be correct only if the synthesis and breakdown of glycogen occurred by two distinct processes and if there were a true pool of glycogen in which incorporated radioactive units immediately became strongly diluted The first assumption is obviously invalid if phosphorylase is involved in both the formation and rupture of the 1 4 α-glucosidic linkages in glycogen Even assuming as has lately been suggested that phosphorylase acts only in glycogenolysis and that another enzyme system is responsible for the synthesis of glycogen it is highly probable that the newly incorporated glucosyl units will be added to the outer tiers of the glycogen molecule therefore at a site where they will be most vulnerable to the action of phosphorylase Any acceleration of glycogenolysis will therefore effectively counteract an increased formation of radioactive glycogen from ^{14}C glucose unless the rearrangement of the glycogen molecules by branching processes is an extremely rapid phenomenon That such is not the case has been demonstrated by Stetten and Stetten[9] and confirmed in our laboratory An example of this is shown in Fig 1 labelled glucose units incorporated under the influence of insulin are seen to disappear very rapidly from the glycogen as conditions become less favourable

In short whether glycogen synthesis is measured chemically or by the incorporation of radioactive glucose the same precautions must be strictly adhered to in experiments designed to explore the role played by insulin in the *physiological* conversion of glucose to liver glycogen

(a) The insulin used must be entirely free of glucagon

(b) The animals must be kept at a normal or slightly elevated blood-sugar level by an exogenous supply of glucose during the whole experimental period since this is the state in which the physiological secretion of insulin takes place

As has been reported in previous publications (Berthet *et al* [1] de Duve[3]) a significant stimulation of hepatic glycogen synthesis by administered insulin has been repeatedly evidenced under the above conditions both by the chemical and by the isotopic methods It has further been shown that the extra glycogen synthetized under the influence of insulin is derived directly from circulating glucose and not from metabolites such as lactate or pyruvate formed in increased amounts by the peripheral tissues Finally an *in vitro* effect of insulin on hepatic glycogen synthesis has been demonstrated on isolated liver slices

An almost perfect counter-demonstration of these results is provided by the recently published data of Spiro *et al* [8] who have shown that the hepatic glycogen stores as well as the ability of the isolated tissue to convert glucose to glycogen decrease rapidly in insulin-treated alloxan-diabetic rats as a result of insulin deprivation and are then restored by the administration of insulin

In view of these results and of numerous previous ones we see no reason at the present time to alter our view that insulin plays an essential and direct role in the physiological deposition of alimentary glucose as liver glycogen The fact that an action of insulin can be demonstrated on muscle or adipose tissue under conditions where no hepatic effects of the hormone are seen is in our opinion irrelevant to the main discussion since the liver occupies a unique metabolic position shared only to some extent with the kidneys as a glucose-forming organ Neither do we see an objection in the well-documented finding (see for instance Winternitz *et al* [10]) that the formation of liver glycogen can be influenced independently of insulin by adrenal steroids The effect of the latter obtains with a variety of precursors and obviously differs in its mechanism from that of insulin which appears to be fairly specific.

Recent investigations carried out by Dr P Jacques in our laboratory have provided some additional information on the action of insulin on isolated liver slices reported by Berthet *et al* [1] The results obtained on glycogen formation in these new experim

are summarized in Table I It will be noticed that a significant effect of insulin is obtained with glucose but not with fructose as precursor and that this effect is maximal between the 60th and 90th minutes of incubation

A careful statistical examination of the results after 30 minutes indicates that the value of 10 5 per cent is not truly representative of the insulin effect but arises from the summation of a large number of experiments in which the hormonal effect was negligible with a limited number of cases where the insulin stimulation ranged between 60 and 150 per cent The proportion of negative results appeared

TABLE I

EFFECT OF ADDED INSULIN ON SYNTHESIS OF GLYCOGEN ^{14}C BY ISOLATED RABBIT LIVER SLICES

Experimental conditions of Berthet *et al* [1] unpublished results of P J cques

Substrate	*Incubation time min*	*Number of comparisons*	*Percentage stimulation by insulin*	*t*	*P*
Glucose-^{14}C	30	91	+10 5 ± 3 1	3 40	0 001
	60	21	+23 7 ± 5 4	4 40	<0 001
	90	14	+24 9 ± 9 3	68	0 0
	1 0	7	+21 0 ± 11 6	1 81	—
Glucose-^{14}C + fructose	30	19	+10 6 ± 4 1	2 60	<0 05
Fructose-^{14}C	30	30	+ 2 7 ± 4 3	0 63	—
Fructose-^{14}C + gluco e	30	23	+ 2 4 ± 6 0	0 40	—

to be smaller in the longer term experiments but would presumably increase again if the incubation were extended over more than _ hours (see Fig 1)

Dr Jacques has also studied the conversion of glucose and fructose carbon to CO_2 in a limited number of experiments His results are shown in Table II As is seen there are indications of a stimulation by insulin of the oxidation of the first glucose carbon and of fructose In all these experiments a significant positive correlation was observed between the effects exerted by insulin on the formation of glycogen and on the production of CO_2 by the same liver slice This correlation was particularly striking ($P < 0\ 001$) when mixtures of the two hexoses with either one or the other labelled were used as substrates

The first conclusion to be drawn from these new results is that the observed effects of insulin are often of borderline magnitude and can easily escape detection unless carefully paired slices are used and the incubation is stopped at an appropriate time This however does not necessarily mean that they are artifacts devoid of physiological significance The results of Fig 1 which were all obtained on slices from the same liver should suffice to render such a hypothesis very unlikely Besides illustrating clearly the importance of time and the preferential effects of insulin on the conversion of

TABLE II

EFFECT OF ADDED INSULIN ON FORMATION OF $^{14}CO_2$ BY ISOLATED RABBIT LIVER SLICES

U p bl shed results f P Jacq es.

S bstrate	I cubat t m m	N mber of mparisons	P tage st m lat on by insul n	t	P
Gl cose-^{1} C	30	20	+ 3 3 ± 5 5	0 6	—
Glucose 1-^{14}C	30 60	6 6	— 3 3 ± 6 4 +23 ± 9	0 52 2 56	— 0 05
Glucose-^{14}C + fructose	30	20	+ 3 9 ± 4 6	0 85	—
Gl cose-1-^{14}C + fru t se	30	6	+ 9 5 ± 4 5	2 11	<0 10
Fructose-^{14}C	30	11	+23 7 ± 10 7	2 20	0 05
Fructose- ^{4}C + gluc se	30	8	+ 3 4 ± 23	0 15	—

glucose to glycogen these results indicate further that a mild treatment of the hormone with alkali which suppresses its hypoglycaemic action also destroys its ability to influence glycogen synthesis in isolated liver slices

A more reasonable conclusion suggested by the experimental facts is that two factors one present in fresh slices and decreasing in efficiency as the incubation proceeds the other appearing during incubation combine to prevent the effect of insulin from becoming evident *in vitro* Only when the combined influences of the two factors are minimal i e on an average between the 60th and 90th

are summarized in Table I It will be noticed that a significant effect of insulin is obtained with glucose but not with fructose as precursor and that this effect is maximal between the 60th and 90th minutes of incubation

A careful statistical examination of the results after 30 minutes indicates that the value of 10 5 per cent is not truly representative of the insulin effect but arises from the summation of a large number of experiments in which the hormonal effect was negligible with a limited number of cases where the insulin stimulation ranged between 60 and 150 per cent The proportion of negative results appeared

TABLE I

EFFECT OF ADDED INSULIN ON SYNTHESIS OF GLYCOGEN-^{14}C BY ISOLATED RABBIT LIVER SLICES

Experimental conditions of Berthet *et al* [1] unpublished results of P Jacques

Substrate	*Incubation time min*	*Number of comparisons*	*Percentage stimulation by insulin*	*t*	*P*
Glucose ^{14}C	30	91	+10 5 ± 3 1	3 40	0 001
	60	21	+23 7 ± 5 4	4 40	<0 001
	90	14	+ 4 9 ± 9 3	68	0 0
	120	7	+21 0 ± 11 6	1 81	—
Glucose-^{14}C + fructose	*30*	*19*	+10 6 ± 4 1	2 60	<0 05
Fructose-^{14}C	30	30	+ 2 7 ± 4 3	0 63	—
Fructose-^{14}C + glucose	30	3	+ 2 4 ± 6 0	0 40	—

to be smaller in the longer term experiments but would presumably increase again if the incubation were extended over more than 2 hours (see Fig 1)

Dr Jacques has also studied the conversion of glucose and fructose carbon to CO_2 in a limited number of experiments His results are shown in Table II As is seen there are indications of a stimulation *by insulin of the oxidation of the first* glucose carbon and of fructose In all these experiments a significant positive correlation was observed between the effects exerted by insulin on the formation of glycogen and on the production of CO_2 by *the same liver slice* This correlation was particularly striking ($P < 0.001$) when mixtures of the two hexoses with either one or the other labelled were used as substrates

account for the increased synthesis of glycogen and oxidation of the first carbon of the glucose molecule as well as for the general correlation between glycogen formation and CO production

As shown in experiments performed in the absence of insulin an increased utilization of unlabelled glucose by the liver slices would tend to depress the formation of radioactive glycogen and to increase the production of $^{14}CO_2$ from radioactive fructose Thus the observed stimulation of the latter process by insulin could be explained simply as a consequence of the effect on glucose utilization but if this were the case one should expect a negative correlation between the effects of insulin on glycogen and CO_2 formation from fructose As mentioned earlier the correlation is positive and it appears therefore that some direct effect of insulin on the utilization of fructose also takes place Possibly as has been observed on the isolated rat diaphragm (Hers~ Mackler and Guest) this effect may be restricted to a pathway of fructose uptake mediated by hexokinase

In conclusion the results described in this paper are in line with those of other authors who have described effects of insulin on fatty acid and protein synthesis in isolated liver slices They provide additional evidence that insulin stimulates directly the uptake of glucose by the liver at least under some conditions

REFERENCES

1 BERTHET J JACQUES P HERS H. G & DE DUVE, C (1956) *Biochim biophys Acta* 20 190
2 BOUCKAERT J P & DE DUVE, C (1947) *Physiol Rev* 27 39
3 DE DUVE C (1956) *Ciba Found Sci Coll Endocrin* 9 203
4 DE DUVE, C HERS H G & BOUCKAERT J P (1946) *Arch int Pharmacodyn* 72 45
5 HERS H G (1955) *J biol Chem* 214 373
6 LEVIN H W & WEINHOUSE, S (1958) *J biol Chem* 232 749
7 MACKLER B & GUEST G M (1953) *Proc Soc exp Biol Med* 83 327
8 SPIRO R C ASHMORE, J & HASTINGS A B (1958) *J biol Chem* 230 761
9 STETTEN M R & STETTEN DEW J (1954) *J biol Chem* 207 331
10 WINTERNITZ, W W DINTZIS R & LONG C N (1957) *Endocrinology* 61 724.

minutes of incubation under the conditions of our experiments are the influences of insulin clearly observable Quite possibly this may never happen under other experimental conditions

We do not know what these two factors may be As far as the first one is concerned it is possible since fed animals were used that the tissue used is practically saturated with insulin at the start and

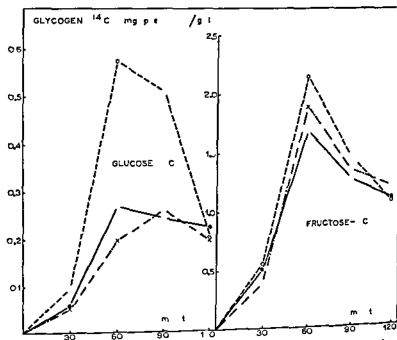


FIG 1 Incorporation of labelled hexoses into the glycogen of isolated rabbit liver slices
●——● Controls
○ ○ Incubated with insulin (1 U/ml)
×—— ——× Incubated with insulin previously inactivated by a 3 hour incubation at 37 in 0 1 N KOH

that the hormone present in the control slices must first become inactivated for a differential effect to become evident A deterioration of the insulin-responsive enzyme systems with time could account for the second effect However there are many alternative explanations and nothing definite can be said at the present time

As to the nature of the insulin effect it is best explained in terms of a stimulation of the primary phosphorylation of glucose or of a transport phenomenon preceding this process since this would

INFLUENCE OF INSULIN ON THE GLUCOSE METABOLISM OF THE LIVER

F Tarding and P Schambye

Novo Terapeutisk Laboratorium Copenhagen

In previous studies of the glucose turnover in the body the single injection of ^{14}C-labelled glucose has been applied[1] as the rate of glucose release and uptake can be calculated from the exponential decrease of the plasma glucose specific activity However it is conditional that the release of glucose into and the uptake of glucose from the extracellular pool is maintained in a steady state Thus it will not be possible to obtain reliable information about the influence of insulin upon the glucose metabolism from such data as the steady state is outbalanced as soon as the hormone is administered and the plasma glucose concentration does not any longer represent the concentration in the various compartments of the pool

In order to be able to measure the rate of glucose release from the liver even during actual changes in the size of the glucose pool a method has been worked out in which the pool size does not enter into the calculations As the specific activity of plasma glucose in portal and arterial blood is identical in the fasting state the amount of unlabelled glucose released by the liver can be calculated by setting the ratio of the specific activity of arterial plasma glucose to hepatic venous glucose equal to the ratio of the amount of glucose passing through the liver to the total amount leaving the liver

This method is applied to a study of the influence of intraportally and intravenously administered insulin upon the release of glucose from the liver

Materials and Methods

The experiments were performed on normal cyclopropane anaesthetized dogs which received their last meal 18 hours before A hepatic vein was catheterized under fluoroscopic control and infusion of Bromsulphalein (BSP) was applied in order to estimate the hepatic plasma flow[2] After an equilibration period following the

If γA and γH represent the specific activity of glucose in arterial and hepatic venous blood the ratio $\gamma A/\gamma H$ will be equal to the ratio between the amount of glucose leaving the liver (Q) and the amount passing through the liver (D) hence

$$\frac{\gamma A}{\gamma H} = \frac{Q}{D}$$

While D cannot be ascertained the value of Q is calculated from the estimated hepatic blood flow and the hepatic-venous glucose concentration and further as

$$Q = D + a$$

where a is the amount of unlabelled glucose released from the liver this amount can be derived as

$$a = Q\left(1 - \frac{\gamma H}{\gamma A}\right) \qquad (1)$$

This equation will be suitable for liver output calculations even in periods where a steady state with respect to glucose metabolism is not maintained as variations in the glucose pool do not interfere

The glucose output calculated according to this procedure is shown in Fig 3 and Table I together with the net amount of plasma glucose leaving the splanchnic organs calculated from the difference in glucose concentration between a hepatic vein and the femoral artery multiplied by the estimated hepatic plasma flow

Results

In Fig 2 the data presented was obtained in a normal dog which was infused intraportally and 14 days later intravenously with 0 08 i u /kg /hr of glucagon free insulin The drop in blood sugar was significantly smaller when the hormone was administered via the portal vein. This observation confirms the findings of Weisberg *et al* [8] who also showed that a content of glucagon in the insulin preparation could not account for the observed difference They explained this phenomenon by assuming that the liver or more specifically the insulinase in the liver retains or inactivates a greater part of the insulin which is administered via the portal vein

The glucose output of the liver before and during an intraportal infusion of 0 02 i u of insulin/kg /hr is shown in Fig 3 Although this dose of insulin does not have any apparent effect upon the arterial blood-sugar concentration it could be anticipated that if

intravenous injection of a dose of randomly labelled ^{14}C glucose (200 μc) blood samples were collected simultaneously from the hepatic vein and the femoral artery every 15 minutes for 1-2 hours which served as a control period Infusions of glucagon free insulin were then started at rates varying from 0 02-0 12 1 u /kg /hr either into a peripheral vein or the portal vein into which a catheter had been inserted via a small jejunal vein at least 4 days prior to the experiments

Plasma glucose was determined according to Nelson[1-] and Somogyi[16] and BSP concentration according to Gaebler[7] and

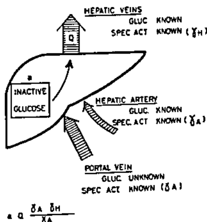


FIG 1 The principle of the isotope dilut on method worked out for calculating the release of unlabelled glucose (a) from the liver (Q = total amount of glucose leaving the liver cf text)

Munkner[11] Determinations of the glucose specific activity were performed as previously described[18]

The principles of the calculation of the amount of unlabelled glucose released from the liver are shown schematically in Fig 1 The specific activity of the glucose in portal and in arterial blood is identical if no dilution with unlabelled glucose absorbed from the intestines occurs This is confirmed experimentally in normal dogs starved for 18 hours although the concentration of glucose may differ in blood from these two vessels The arterial specific activity can thus be regarded as representative for all blood glucose entering the liver

insulin affects the glucose uptake of the liver cells this dose given intraportally might be followed by an alteration in the net amount of glucose leaving the liver and also in its release of unlabelled glucose This however was not the case as the values remained approximately constant for the whole infusion period although the hepatic cells presumably were exposed to concentrations of insulin which would stimulate the glucose uptake of the peripheral tissues

Similar results were obtained with intraportal infusions of 0 04 0 08 and 0 12 i u /kg /hr In Table I the arterial and hepatic-venous plasma glucose and its specific activity are listed together with the calculated plasma output from the liver of unlabelled glucose before and during an infusion of 0 12 i.u /kg /hr intraportally The decrease in the arterial blood sugar only indicates an increased uptake of glucose in the peripheral tissues as the output from the liver remained almost constant

TABLE I

EFFECT OF PORTAL INFUSION OF INSULIN (0 12 I.U /KG /HR.) UPON THE ARTERIAL AND HEPATIC-VENOUS PLASMA GLUCOSE CONCENTRATIONS AND THE OUTPUT OF UNLABELLED GLUCOSE FROM THE LIVER OF A NORMAL DOG

Min from start of infusion	Femoral artery mg glucose/ 100 ml plasma	Femoral artery c p.m /mg glucose × 10⁻³	Hepatic vein mg glucose/ 100 ml plasma	Hepatic vein c p.m /mg glucose × 10⁻³	Plasma glucose output† mg /min
−75	121	323	138	2[illegible]6	39
−60	115	82	139	232	41
−45	116	238	137	197	37
−30	117	197	138	166	34
−15	111	169	141	142	33
2	104	147	141	123	33
16	96	124	134	100	39
31	84	105	118	80	43
45	73	84	100	69	29
60	67	66	92	55	3
75	60	57	81	45	32

The hepatic plasma flow averaged 150 ml./min.
† Calculated from the equation.[1]

DISCUSSION

The results obtained after intraportal infusion of relatively small doses of glucagon free insulin into cyclopropane anaesthetized normal dogs indicate that the glucose output from the liver is not, even partly inhibited by the influence of exogenous insulin. The

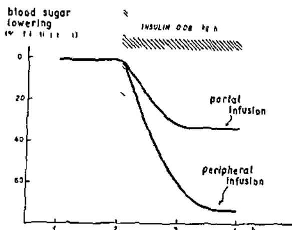


FIG 2 Comparison of the hypoglycaemic effect of intraportally and intravenou ly infused insulin into the same normal anaesthetized dog

- - - Specific Activity
—— Glucose concentration
Hepatic vein
Femoral artery

GLUCOSE OUTPUT

Isotop d l t n meth d L cain t r sat on t ch iq

FIG 3 Effect of intraportally infused in ulin (o o 1 u/kg/hr) into a normal dog upon the output of unlabelled glucose from the liver and the net amount of glucose leaving the splanchnic organs In the upper part of the figure re shown the arterial and hepatic venous plasma gluco e concentration and specific activity

carried out in animals in the post-absorptive state in which the release of glucose from the liver has been the dominating process responsible for the upkeep of a constant blood sugar In addition insulin has nearly always been administered intravenously or subcutaneously and thus been offered the change of being bound to or affecting the peripheral tissues far more than the liver and thereby shifting the whole glucose transport into a more pronounced one-sided process in the direction from the liver to these tissues than before

Future experiments probably have to be designed so the influence of insulin can be investigated upon the liver of intact animals which are not only receiving physiological amounts of insulin via the portal venous blood but also are maintained in a state where the glucose metabolism has what might be defined as an inward hepatic trend

Summary

1 The inadequacy of the isotope dilution method after a single injection of ^{14}C glucose for measuring smaller variations in the glucose release from the liver during changes in the size of the glucose pool prompted the development of a method which permitted the calculation of the amount of unlabelled glucose released by the liver at any time during changes in the plasma glucose concentration This was done by relating the specific activity of hepatic-venous and arterial blood sugar to the directly measured total amount of glucose leaving the splanchnic system

2 This method was applied in studying the effect of intraportal and intravenous insulin infusion of from 0 02-0 12 i u /kg /hr into normal dogs As previously observed the hypoglycaemic effect obtained after intraportal infusion is 40-50 per cent smaller than after intravenous infusion of an identical dose but no decrease in the hepatic glucose output was found

3 It is suggested that future experiments should be designed so that the effect of intraportally administered insulin is studied in a liver which is already taking up glucose

The authors wish to express their appreciation to Dr K Hallas-Møller for valuable suggestions and criticism and their indebtedness to Professor Aa Thordahl-Christensen D V M the Royal Veterinary and Agricultural College Copenhagen for placing the X ray equipment of his department at their disposal

investigation which combines the physi ' introducing insulin into the blood with mea ' of glucose released from the liver alone, should sidered in relation to previous experimental insulin effect upon the liver was studied in o'

Many investigators have not been able to effect upon the glucose uptake or release of the ' fusion experiments[9 13 14] in liver slices[15] or intact the liver was suddenly excluded from the blood

On the other hand a decrease in the glucose ' by liver catheterization of patients[1] or with an technique using either a single injection[4] or a co ^{14}C glucose [19] although the authors of the latter of the decrease in the blood sugar is predominantly peripheral glucose uptake However, our studies with did not show any evidence of an inhibition of the release of 0 15 i u of insulin/kg were injected intravenousl Berthel *et al* [2] found that insulin *in vitro* stimulates the *deposition of radioactive glucose into the glycogen of'*

None of these results present the necessary conclusive nor does the work which is characterized by the co ministration of glucose to obviate the fall in blood insulinized preparation In such dogs de Duve *et al* [5] fou the necessary amount of glucose was reduced to about by hepatectomy while Lundsgaard[10] could not confirm this o tion in cats Lang *et al* [8] found in dogs an almost similar d as de Duve although they interpreted this to be due to a factor which stimulated the peripheral utilization of gl did not believe that the utilization of the liver was causin difference

The interesting data of Cahill *et al* who demonstrated a free meability of the liver cell membrane with respect to glucose cluded any effect of insulin upon the transport of glucose across hepatic cell membrane but suggested that the rate at least determined by the activity of glucokinase, glucose-r the size of the glucose-6-phosphate pool and the level of circulatin glucose If the hormone does have an effect on these factors thereby on the further transport of glucose into the metabolic path ways then it should be studied under such circumstances that the liver cells already take up glucose Previous work has mainly '

DISCUSSION

MAHLER: We have attempted, in as direct a physiological manner as possible, to solve the problem of the immediate effect of insulin on glucose production by the liver. Plastic catheters were placed at operation in the

TABLE I

MEANS OF RESULTS IN NINE NORMAL DOGS

	Control period	Minutes after injection of insulin			
		5	10	15	30
Hepatic plasma flow (ml./min./kg.)	30.2	32.4	37.4	28.4	34.0
Difference between hepatic and portal plasma glucose (mg./100 ml.)	9.9	13.7	13.7	10.7	18.4
Hepatic glucose output (mg./min./kg.)	3.1	4.3	5.1	3.1	6.8

portal and hepatic veins of normal dogs a few days before any further experiments were undertaken. In this way it was possible to obtain blood entering and leaving the liver at any instant without the metabolic disturbances of acute surgery and anaesthesia.

TABLE II

EFFECT OF INSULIN ON RECYCLING OF ^{14}C

Recycling is expressed as the percentage of ^{14}C appearing in carbon atom 1 of plasma glucose after the injection of a tracer dose of glucose labelled at carbon atom 6.

Time from injection of 6-^{14}C glucose	Recycling of labelled carbon atom		
	Per cent of plasma glucose specific activity		
Minutes	Dog C-38	Dog C-39	Dog C-41
15	3.8	—	0
25	6.2	5.0	—
35	7.2	4.4	5.2
	Injection of insulin		
40	17	7.2	16
45	18.8	11.8	12
50	19	16.8	—
65	35	—	40

Hepatic glucose production, the amount of glucose added by the liver to the blood flowing into it, could then be calculated from the hepatic blood flow and the difference in glucose concentration in hepatic and portal vein plasma.

REFERENCES

1 BEARN A G BILLING B H & SHERLOCK, S (1952) *Clin Sci* 11 151
2 BERTHET J JACQUES P HENNEMAN G & DE DUVE C (1954) *Arch I ternat Physiol* 62 282
3 BRADLEY S E INGLEFINGER F J BRANDLEY G P & CURRY J J (1945) *J Cl n Invest* 24 890
4 DUNN D F FRIEDMANN B MAASS A R REICHARD G A & WEINHOURSE, G (1957) *J biol Chem* 225 225
5 DE DUVE C DENAYER P P OOSTVELD M & BOUCKAERT J P (1945) *Arch Internat Pharmacodyn* 70 78
6 FELLER D D CHARKOFF I L STRISOWER E H & SEARLE C L (1951) *J biol Chem* 188 865
7 GAEBLER O H (1949) *Amer J Cl n Pathol* 15 452
8 LANG S GOLDSTEIN H S & LEVINE, R. (1954) *Amer J Phys ol* 177 447
9 LUNDSGAARD E NIELSEN N A & ORSKOV S L (1936) *Skand Arch Physiol* 73 296
10 LUNDSGAARD E (1954) *Acta Physiol Scand* 31 215
11 MUNKNER T (in the Press)
12 NELSON N (1944) *J biol Chem* 153 175
13 NIELSEN N A (1933) *Skand Arch Physiol* 66 1
14 NIELSEN N A (1933b) *Skand Ar h Phys ol* 66 19
15 RENOLD A HASTINGS A B NESBETT F B & ASHMORE J (1955) *J b ol Chem* 213 135
16 SOMOGYI M (1945) *J biol Chem* 160 61
17 STEINCKE, K (1951) Thesis *Leverens Gl kose fgift under Insulinpdv rkning* Dansk Videnskabs Forlag Copenhagen
18 TARDING F & SCHAMBYE P (1958) *Endokrinolo ie* 36 2 2
19 WALL J S STEELE, R DE BODO R C & ALTSZULER N (1957) *Amer J Ph ysiol* 189 51
20 WEISBERG H F FRIEDMAN A & LEVINE R (1949) *Amer J Phys ol* 158 332

DISCUSSION

MAHLER We have attempted in as direct a physiological manner as possible to solve the problem of the immediate effect of insulin on glucose production by the liver Plastic catheters were placed at operation in the

TABLE I

MEANS OF RESULTS IN NINE NORMAL DOGS

	C trol pe od	M te after j t on of insi l			
		5	10	15	30
Hepatic plasma fl w ml /mm / kg	30 2	32 4	37 4	28 4	34 0
Differenc between hepatic and p rt l pl ma glucose mg / 100 ml	9 9	13 7	13 7	10 7	18 4
Hepatic glucose outp t mg / mın /kg	3 1	4 3	5 1	3 1	6 8

portal and hepatic veins of normal dogs a few days before any further experiments were undertaken In this way it was possible to obtain blood entering and leaving the liver at any instant without the metabolic disturbances of acute surgery and anaesthesia

TABLE II

EFFECT OF INSULIN ON RECYCLING OF[1] C

Recycling is expressed as the percent ge of[1] C appearing in c bon atom 1 of pla ma glucose after the injection of a tr cer dose of glu ose l bell d at carbon at m 6

T me f nj ct o of 6 [1] C gluco e	R cy l g of l a t e carb toms		
	Per ce t f pl sma gl e pe fic ct ty		
M n tes	D g C-38	D g C 39	D g C-43
15	3 8	—	0
25	6 2	5 0	—
35	7 2	4 4	5.2
	I ject o f nsul		
40	17	7.2	16
45	18 8	11 8	12
50	19	16 8	—
65	35	—	40

Hepatic glucose production the amount of glucose added by the liver to the blood flowing into it could then be calculated from the hepatic blood flow and the difference in glucose concentration in hepatic and portal vein plasma

REFERENCES

1 Bearn A G Billing B H & Sherlock S (1952) *Clin Sci* 11 151
2 Berthet J Jacques P Henneman G & de Duve C (1954) *Arch Internat Physiol* 62 282
3 Bradley S E Ingelfinger F J Brandley G P & Curry J J (1945) *J Clin Invest* 24 890
4 Dunn D F Friedmann B Maass A R Reichard G A & Weinhouse G (1957) *J biol Chem* 225 225
5 de Duve C Denayer P P Oostveld M & Bouckaert J P (1945) *Arch Internat Pharmacodyn* 70 78
6 Feller D D Charkoff I L Strisower E H & Searle C L (1951) *J biol Chem* 188 865
7 Gaebler, O H (1949) *Amer J Clin Pathol* 15 452
8 Lang S Goldstein H S & Levine R (1954) *Amer J Physiol* 177 447
9 Lundsgaard E Nielsen N A & Ørskov S L (1936) *Skand Arch Physiol* 73 296
10 Lundsgaard F (1954) *Acta Physiol Scand* 31 215
11 Munkner T (in the Press)
12 Nelson N (1944) *J biol Chem* 153 175
13 Nielsen N A (1933a) *Skand Arch Physiol* 66 1
14 Nielsen N A (1933b) *Skand Arch Physiol* 66 19
15 Renold A Hastings A B Nesbett F B & Ashmore J (1955) *J biol Chem* 213 135
16 Somogyi M (1945) *J biol Chem* 160 61
17 Steincke K (1951) Thesis *Leverens Glukose-fyft under Insulinpåvirkning* Dansk Videnskabs Forlag Copenhagen
18 Tarding F & Schambye, P (1958) *Endokrinologie* 36 222
19 Wall J S Steele R de Bodo R C & Altszuler N (1957) *Amer J Physiol* 189 51
20 Weisberg H F Friedman A & Levine R (1949) *Amer J Physiol* 158 332

DISCUSSION

MAHLER We have attempted in as direct a physiological manner as possible to solve the problem of the immediate effect of insulin on glucose production by the liver Plastic catheters were placed at operation in the

TABLE I

MEANS OF RESULTS IN NINE NORMAL DOG

	C trol per od	M n tes after ject on of sul n			
		5	10	15	30
H p t c pl sma flow ml /min / kg	30 2	32 4	37 4	28 4	34 0
D fference betwee h patic and portal pla ma gl se mg / 100 ml	9 9	13 7	13 7	10 7	18 4
H pat gluc se output mg / n m /kg	3 1	4 3	5 1	3 1	6 8

portal and hepatic veins of normal dogs a few days before any further experiments were undertaken In this way it was possible to obtain blood entering and leaving the liver at any instant without the metabolic disturbances of acute surgery and anaesthesia

TABLE II

EFFECT OF INSULIN ON RECYCLING OF ^{14}C

Recycling is exp sed a the percent ge of C appearing in c bon atom 1 of plasma glucose fter th injection of a t dose of gl cose l b ll d at carb n atom 6

T m f m i j ct o f 6-14 C gl	Re y l g of r d a t ve carb toms		
	Pe cent f plasma gl pec fic ct ty		
M te	D g C 38	D g C-39	D g C 43
15	3 8	—	0
25	6 2	5 0	—
35	7 2	4 4	5 2
I j ct o f ns l			
40	17	7 2	16
45	18 8	11 8	12
50	19	16 8	—
65	35	—	40

Hepatic glucose production the amount of glucose added by the liver to the blood flowing into it could then be calculated from the hepatic blood flow and the difference in glucose concentration in hepatic and portal vein plasma

Before the injection of insulin the difference in glucose concentration between the hepatic and portal vein was 9 9 mg per 100 ml and hepatic glucose production 3 1 mg per minute per kg body weight Immediately after the injection of 0 2 units per kg of glucagon free insulin glucose concentration dropped in both the hepatic and portal veins However the concentration gradient between them did not change significantly and there was no decrease in hepatic glucose production In fact the

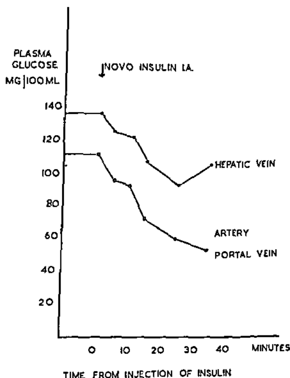


FIG 1 The effect of a single injection of glucagon free insulin on *glucose concentrat on.* (Dog C-32)

only significant change was an increase in glucose production in *response* to the hypoglycaemia *when the portal* glucose concentration had dropped to 60 mg per 100 ml or less

The fall in blood-sugar concentration in normal dogs in *response to* insulin is therefore *not due to any effect* of insulin on glucose production by the liver but results from an increase in glucose utilization by insulin sensitive tissues

From the reversal after insulin of the glucose concentration difference between the artery and portal vein it is evident that the tissues of the splanchnic area which include the mesenteric fat take part in this response Consequently calculations of hepatic glucose output based on concentration differences between hepatic vein and artery give misleading results (Bearn *et al* *Clin Sci* 1952 11 151)

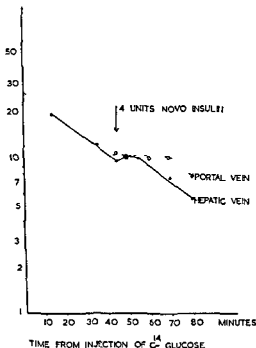


FIG 2 Cha ge in p cific ctivity of plasma gluco bef e and after n injecti n of insulin (D g C-32)

After the injection of a tracer dose of [1] C-labelled glucose its specific activity in the plasma decays at an exponential rate

The injection of insulin is immediately followed by a *change in the* rate of decay for a variable period of time In some experiments there appears to be a plateauing of the curve and this has been interpreted by some (Dunn *et al* *J Biol Chem* 1957 2.5 .25) to indicate complete inhibition of hepatic production

however a qualitative change in the source of the glucose coming from the liver a portion of it is now derived from glucose metabolites coming from insulin sensitive tissues flooding the liver and being reconverted in the liver to glucose In other words there is a speeding up of the Cori cycle

LEVINE I should like to show some unpublished data of work which Dr Steele Dr Bishop and I did at the Brookhaven Laboratory this summer

There are numerous publications now in the literature on the use of an injection of C glucose in order to measure the output of C glucose by the liver The specific activity of the plasma glucose is determined and the log of the specific activity is plotted against time The specific activity decreases due to the dilution by C glucose continuously delivered by the liver Weinhourse Wrenshall Searle and others have shown that the injection of insulin and/or the administration of a single large dose of ^{12}C glucose stops the further descent of the specific activity curve This plateau has been interpreted as showing that no further unlabelled glucose is now coming into the pool Thus glucose and insulin are said to cause an inhibition of glucose output presumably from the liver

Dr Steele and Dr Bishop recently did such experiments with one amendment We used liverless dogs The animals received a constant injection of C glucose from a pump so that the ^{1} C glucose continuously delivered could not be inhibited by hormonal influences When unlabelled glucose was given a plateau lasting about 25-30 minutes was obtained Whatever may be the true explanation it surely cannot be explained by an inhibition of output of glucose by non-existent liver Insulin did not produce a plateau but neither did it do so in *intact* controls It would seem at present that the effect depends upon something present in certain lots of crystalline insulin

We believe in concert with other experiments by Steele and de Bodo that the glucose plateau is probably due to equilibrium of glucose across body compartments especially between the vascular and extracellular fluid

KRAHL Stimulation of peptide synthesis by insulin added *in vitro* can be demonstrated in liver slices from mildly diabetic rats (Krahl *Rec Progress Hormone Research* 1956 **12** 199) The stimulation is most marked – or 3 days after the induction of diabetes in the liver donor In long-standing severe diabetes enzymatic mechanisms for peptide synthesis are damaged insulin then has no effect *in vitro* but restores the capacity for synthesis after injection and the passage of some hours The *in vitro* insulin effect with liver slices from mildly diabetic rats is related to presence of glucose in the medium for example the radioactivity of glycine-1 ^{1} C incorporated into the peptide glutathione was found to be for

control slices from mild diabetics 63‿ c p m per mg glutathione with insulin but no glucose 598 with glucose but no insulin 1480 with insulin plus glucose ‿940

So in summary these are experiments which suggest that under a rather special set of conditions insulin does have an *in vitro* effect on peptide synthesis in liver slices and that this particular effect is due to the effect of insulin on the use of glucose Later today both Professor Young s group and I will have something to say about the effect of insulin in the absence of glucose on peptide synthesis

CORI Thank you Professor Krahl

LONG What I have to contribute to this subject is negative in the sense that it is not directly concerned with the effect of insulin on liver glycogen Professor de Duve recalled to your mind that there are other hormones which have a very potent effect in stimulating liver glycogen formation These of course are the adrenal steroids of the type of cortisone and hydrocortisone

I think we all appreciate that these materials when given to fasted animals bring about a rapid increase in the amount of glycogen in the liver I think we should all know and it has been demonstrated many times that this effect is not confined entirely to glycogenosis from the products of increased protein breakdown in the muscles It has been shown by several investigators including ourselves that if a measured amount of glucose is given to animals with and without the adrenal steroids that in the presence of adrenal steroids a larger proportion of glucose is recovered as liver glycogen In other words there is a stimulation of glycogen synthesis not only from the intermediary metabolites of protein catabolism but from glucose itself

The other point that may b mentioned in this regard is the observation made by several laboratories that in severely alloxan-diabetic fasted rats the level of liver glycogen is much higher than that observed in normal rats fasted to a comparable degree This we have observed and it is of course abolished as many investigators have shown by adrenalectomy

The point then arises as to whether these effects of the adrenal steroids on liver glycogen formation have any relation to the presence or absence of insulin in the body There is a certain amount of evidence that they are not related to the presence of insulin Miller for example has shown that in severely alloxan-diabetic rats which are then adrenalectomized the administration of cortisone to such ... ts in the same increase in liver glycogen as is observed in ... are simply adrenalectomized and have of course ... insulin in ... He concluded, therefore that this ... of the ... in su ... glycogen ... sence

A good many years ago when Lukens and I were interested in the effect of adrenalectomy on depancreatized animals on some occasions we sacrificed both adrenalectomized-depancreatized animals and hypophysectomized-depancreatized animals at periods of up to 30 or 40 days after the pancreatectomy when they were eating well and presumably in a reasonable state of health and we also found at that time that the amounts of glycogen in the liver were comparable to those found in normal animals subjected to the same dietary regime and of course very much greater than those found in animals that were in diabetic acidosis and coma

Our general conclusion has been that so far as the stimulation of liver glycogen formation by adrenal cortical hormone is concerned it does not appear to be dependent on the presence of insulin That of course does not say that insulin is not also required for glycogen synthesis under other circumstances

CHAIN I should like to complement Dr Krahl s remarks We have had this experience we have done a number of perfusion experiments with radioactive glucose in liver with a technique which my colleague Bovet has worked out and which gives excellent glycogen synthesis and we find that in normal liver the turnover of glycogen is at maximum so that you could not expect an insulin effect in fact we do not get one In the perfused liver of a mild diabetic animal on the other hand we do find an effect on the glycogen synthesis it is small but it is there

As far as liver slices are concerned we have now had occasion during our nucleotide work to compare the nucleotides in the intact liver in the perfused liver and in slices We find that in the perfused organ about a third of the nucleotides in the intact liver disappear In the liver slices more than two-thirds disappear In view of these results we distrust results obtained with liver slices

I wonder whether this small effect on glycogen synthesis which Professor de Duve has observed on liver slices might not be due to an effect on one particular kind of cell the Kupffer cells the phagocytic cells which may be more resistant than normal liver cells to slicing I think perhaps that could be the explanation and I would be very interested to hear what Profes or de Duve has to say about it We have never been able to find a really significant effect of insulin under those conditions

CORI Thank you Professor Chain Would you like to answer this question Professor de Duve about the Kupffer cell versus the liver cell?

DE DUVE We have not localized the glycogen in our experiments This was done a number of years ago by Dr Hastings and his group who found that the glycogen formed by liver slices is deposited almost exclusively in the peripheral layer of cells that is in the cells which receive the most oxygen

With regard to the other comments made they all sound rather damaging and I feel that if we had shares in the liver most of us would start selling out after what we have heard There is no doubt that the hepatic actions of insulin are elusive and more easily missed than demonstrated As a result of this negative results are much easier to obtain than positive ones and the latter are usually registered only by workers who believe an action is to be found and have the patience to go on looking for it under different conditions Thus personal bias exerts a significant influence on the nature of the results and this is very unfortunate Despite this fact there are now on record at least ten different instances in which effects of insulin have been observed on the synthesis of glycogen fatty acids or proteins in liver slices or perfused livers and these have to be explained away as artifacts devoid of physiological significance before the hepatic action of insulin can be denied

That lack of insulin causes severe disturbances of hepatic metabolism is recognized by everybody and the point at issue is really whether insulin corrects them by a direct and rapid action on the liver or indirectly and in a delayed fashion by way of its effects on other tissues A well known and important paper by Renold *et al* (*J biol Chem* 1955 213 135) has done much to further the indirect theory With Dr Renold s permission, I would like to recall the more recent investigations by Spiro *et al* (*J biol Chem* 1958 230 761) which argue in favour of the direct theory showing that the hepatic disturbances occur as rapidly as the muscular one in insulin-treated alloxan-diabetic rats following insulin deprivation and that they are then corrected in less than 4 hours by the administration of insulin Unfortunately the authors did not study the effect of insulin after a shorter time-span

CORI This is I think a good summary of the whole problem and I wonder whether Dr Renold would add to what has been said

RENOLD Professor de Duve has mentioned the somewhat different results obtained in Dr Hasting s laboratory by Spiro – using insulin treated alloxan-diabetic rats acutely depleted of insulin – and ours lf – using chronic alloxan-diabetic rats given insulin The difference for the time being at least is one of degree not quality Some lag of the liver response when compared with the muscle response was still observed It is furthermore difficult to know when insulin withdrawal for each tissue really begins Finally Spiro s findings do not alter the fact that in chronic alloxan-diabetic rats insulin produces hypoglycaemia and increased muscular glycogen synthesis immediately but does not concurrently alter hepatic glucose uptake or output Hence in this situation at least the hypoglycaemic effect of insulin cannot be secondary to an hepatic effect

I should also like to comment more generally Some years ago when

we reported the differences existing in the response to insulin administration of subsequently isolated tissues we were particularly impressed by the *differences between tissues* rather than with the *absence* of an insulin effect on liver We concluded that *either* insulin did not directly alter liver metabolism *or* that it exerted a different effect upon this tissue The need for the differing nature of such an effect upon liver if any has since become even more apparent since the absence of a permeability barrier for glucose at the surface of the liver cell has become so clearly established Such a different effect of insulin may well exist and it is tempting to suggest that it should perhaps be looked for in the liver as the tissue in which the demonstration of such an effect woud be least likely to be clouded by the more easily obtained effect at the cell surface To be sure the liver cell may have other interphases or membranes than the cell surface A truly different type of insulin effect upon liver has for instance been recently postulated by a group of workers in Munich (Lamprecht *Biochemische Zeitschrift* July 1958) although the presently published evidence is not completely convincing

SMITH I would like if I may just to ask a question Liver contains an enzyme which inactivates insulin and which has been called insulinase Is this a factor which should be taken into consideration in attempts to demonstrate an effect of insulin on carbohydrate metabolism in liver? For example could the inconstancy of the response of the liver slice to insulin be attributable to destruction of added insulin by this enzyme to variable extents in different experiments?

CORI Does anyone want to say anything about the destruction of insulin in the liver and how rapidly it might be in normal liver as compared to diabetic liver Are there any data available? It seems that no one can answer your question I am sorry

SCHAMBYE. As you may imagine from my talk I am not willing to sell out any shares to Professor de Duve yet I think it is very important also to realize the difference between the normal and the diabetic state When we lack insulin completely certainly the liver glucose metabolism is normal and this may show that ordinarily the insulin present in the normal animal keeps up a normal carbohydrate metabolism which we cannot copy in any way by adding insulin either intraportally or intravenously because we have a different direction of events Therefore I suppose that before we really give up our shares we should try to study the effect when the liver cells are working in the same way as the muscle cell does when we try to influence it with insulin

CORI The amount of work now available on the action of insulin on the liver is large indeed but it seems we need many more experiments before this problem can be finally settled

PART V

INSULIN AND PROTEIN SYNTHE

Chairman PROFESSOR F DICKENS

INSULIN AND INCORPORATION OF AMINO ACIDS INTO PROTEIN

K. L. MANCHESTER and F. G. YOUNG

Department of Biochemistry, University of Cambridge

The original discovery of Von Mering and Minkowski in 1889[17] that the fasting depancreatized dog has a much larger negative nitrogen balance than its normal control has long suggested that insulin has an anabolic influence over protein metabolism. More recently, Lukens and McCann[14] have shown that administration of insulin to the depancreatized-hypophysectomized cat leads to the retention of nitrogen, and Best *et al.*[1,11,0] have found that treatment of the hypophysectomized rat with insulin induces growth, though this latter observation has been challenged by Wagner and Scow[23] who claim that insulin only promotes the appetite of the rat, and that similar results can be obtained by forced-feeding. Many workers have noticed[5,7,13] that insulin lowers the blood amino-acid level of the intact animal and suppresses the rise of blood amino acids which normally follows evisceration.[3,4] Insulin lowers the blood level of ^{35}S methionine administered to either the intact or eviscerated dog, so that the fall of the blood amino-acid level following insulin is the result of an increased removal of amino acid from the blood rather than a smaller release of amino acid by the tissues. That insulin depresses the rise of the blood amino-acid level in the eviscerated animal indicates that its site of action must be, at least in part, the extrahepatic tissues.

Lack of a suitable *in vitro* system has so far hampered attempts to disclose in more detail the mechanism of the action of insulin on protein metabolism. In particular, experiments so far reported do not differentiate as to whether the effect of insulin on protein metabolism is mediated through a primary action on glucose utilization or by a direct effect on protein metabolism unrelated to the action of insulin on carbohydrate biochemistry. An investigation of this problem has been the aim of the work described here.

Sinex *et al.* in 195-[21] and Krahl in 1953[9] described experiments in which they studied the incorporation of ^{14}C alanine and glycine

respectively into the protein of the isolated rat diaphragm when it was incubated in a medium containing the labelled amino acid. Both groups reported that the addition of insulin to the medium stimulated incorporation of ^{14}C alanine and glycine in o protein when incubated in a medium containing no added glucose or other carbohydrate. Addition to the medium of glucose in place of insulin however produced varied effects. Krahl found that it stimulated the transfer of ^{14}C glycine into the protein of the diaphragm whereas Sinex *et al* found that addition of glucose to the medium actually depressed incorporation of ^{14}C alanine into protein and that this depression was even more pronounced when pyruvate was added to the medium in place of glucose. Both groups found that addition of glucose or pyruvate to the medium diminished the stimulating effect of insulin on incorporation. In view of the significance of these results it was decided to study further the effect of insulin glucose and pyruvate on incorporation of a large number of different labelled amino acids into the protein of the isolated diaphragm.

Diaphragm was removed from Albino Wistar rats weighing about 130 g which had fasted about 20 hours before death. The hemi-diaphragms were incubated for 2 hours at 37 in a Dubnoff metabolic shaker in a bicarbonate medium gassed with $O_2 + CO$ (95 5) and containing labelled amino acid (1 or 3 μmole/ml and specific activity about 130 μC/m-mole). Glucose and pyruvate when present were added to a concentration of 12 5 μmole/ml (2 5 mg/ml and 1 2 mg/ml respectively). Insulin (Boots crystalline) was used at a final concentration of 0 5 unit/ml. At the end of the incubation the hemi-diaphragms were homogenized in trichloroacetic acid (TCA) washed in hot TCA (to extract nucleic acid) dissolved in NaOH reprecipitated with TCA the precipitate washed with alcohol then ether and dried in a vacuum desiccator. The protein samples were ground to a powder plated and counted as infinitely thick samples. Though no absolute specific activities were determined the counts obtained from different samples were directly proportional to their different specific activities[18].

Isolated diaphragm will incorporate ^{14}C from ^{14}C-labelled amino acids into protein at a constant rate for a period of at least 6 hours even when no readily oxidizable material is simultaneously added to the medium, but circumstances which interfere with energy production (anaerobiosis and metabolic inhibitors such as cyanide azide

and dinitrophenol) severely curtail incorporation (Fig 1) ^{14}C incorporated into the diaphragm appears to be firmly bound – when diaphragm which has been incubated in a medium containing ^{14}C-labelled amino acids is incubated for a further period in a similar medium but containing unlabelled amino acids the ^{14}C in the diaphragm is not lost It is of interest to note that the rate of incorporation of a ^{14}C-labelled amino acid is not changed by the addition of other unlabelled amino acids

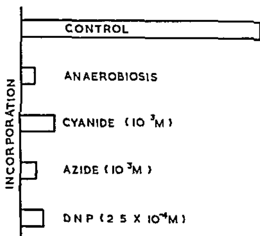


FIG 1 Effect of anaerob osis and of the ddition of va ous inh b tors to the medium o the incorporation of C from 1 C glycine into protein of the isolated rat diaphragm

Addition of insulin to a medium containing no added oxidizable substrate has been found to stimulate the incorporation into protein of the isolated diaphragm of ten different ^{14}C-labelled amino acids of a mixture of ^{14}C-labelled amino acids (a radioactive protein hydrolysate) and of one ^{35}S labelled amino acid (Fig 2) Though the amount of activity incorporated and the magnitude of the stimulation by insulin varied from one amino acid to another in each case addition of insulin to the medium produced a highly significant stimulation of incorporation of labelled amino acid into protein The amount of radioactivity incorporated into protein from glutamate and aspartate was rather low whereas incorporation from leucine in particular was much higher than the general average

On the other hand the response of leucine to the addition of insulin was rather less than average, whereas the stimulation by insulin of incorporation of methionine was much higher than for any other amino acid

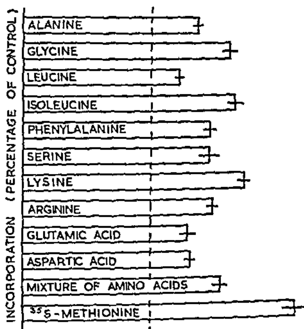


FIG 2 Effect of the addition of insulin to a medium containing no oxidizable substrate on the incorporation of radioactivity from labelled amino acids into protein of the isolated rat diaphragm

The blocks represent incorporation in the presence of insulin incorporation in the absence of insulin is shown by the dotted line All the amino acids were the L-isomers and uniformly ^{14}C-labelled except 1-^{14}C glycine and ^{35}S methionine

Addition of glucose to the medium had no observable effect on the incorporation of most of the different amino acids into the protein of isolated diaphragm (Fig 3) except in the case of methionine leucine and glutamate where a slight stimulation was obtained, and of alanine where a definite depression of incorporation of ^{14}C was evident Addition of pyruvate to the medium likewise had no observable effect on the incorporation of most of the different amino acids into protein of the isolated diaphragm (Fig 4) except possibly in the case of aspartate and again very definitely in the case of alanine.

Alanine apart addition of insulin to a medium already containing

glucose or pyruvate stimulated the incorporation into protein of all the amino acids studied (Figs 5 and 6) and it will be seen by comparison with Fig 2 that the magnitude of the stimulation produced by insulin is the same when either glucose or pyruvate are added to the medium as in their absence In general it appears that the incorporation of a labelled amino acid into the protein of the

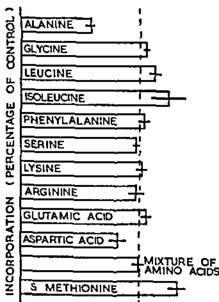


Fig 3 Effect of the addition of glucose to the medium on the incorporation of radioactivity from labelled amino acid into protein of the isolated rat diaphragm.
The blocks represent incorporation in the presence of glucose incorporation in the absence of glucose is shown by the dotted line
All the amino acids were the L-isomers and uniformly ^{14}C-labelled, except 1-^{14}C glycine and ^{35}S methionine

isolated rat diaphragm is not affected by the addition of glucose or pyruvate to the medium but that it is enhanced by the addition of insulin equally in the presence or absence of added glucose or pyruvate

These results are interpreted to mean that insulin stimulates protein synthesis as measured by the transfer of labelled amino acids

On the other hand the response of leucine to the addition of insulin was rather less than average whereas the stimulation by insulin of incorporation of methionine was much higher than for any other amino acid

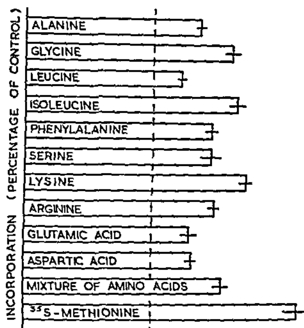


FIG 2 Effect of the addition of insulin to a medium containing no oxidizable substrate on the incorporation of radioactivity from labelled amino acids into p otein of the isolated rat diaphragm

The blocks represent incorporation in the presence of insulin, incorporation in the ab ence of insulin is shown by the dotted line All the amino acids were the L-isomers and uniformly ^{14}C-labelled except 1-^{14}C glycine and ^{35}S methionine

Addition of glucose to the medium had no observable effect on the incorporation of most of the different amino acids into the protein of isolated diaphragm (Fig 3) except in the case of methionine leucine and glutamate where a slight stimulation was obtained and of alanine where a definite depression of incorporation of ^{14}C was evident Addition of pyruvate to the medium likewise had no observable effect on the incorporation of most of the different amino acids into protein of the isolated diaphragm (Fig 4) except possibly in the case of aspartate and again very definitely in the case of alanine

Alanine apart addition of insulin to a medium already containing

glucose or pyruvate stimulated the incorporation into protein of all the amino acids studied (Figs. 5 and 6) and it will be seen by comparison with Fig. 2 that the magnitude of the stimulation produced by insulin is the same when either glucose or pyruvate are added to the medium as in their absence. In general it appears that the incorporation of a labelled amino acid into the protein of the

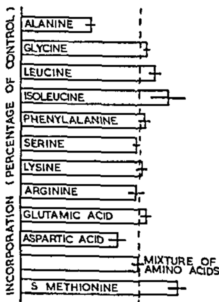


FIG. 3. Effect of the addition of glucose to the medium on the incorporation of radioactivity from labelled amino acids into protein of the isolated rat diaphragm.
The blocks represent incorporation in the presence of glucose; incorporation in the absence of glucose is shown by the dotted line.
All the amino acids were the L-isomers and uniformly ^{14}C-labelled except 1-^{14}C glycine and ^{35}S methionine.

isolated rat diaphragm is not affected by the addition of glucose or pyruvate to the medium but that it is enhanced by the addition of insulin equally in the presence or absence of added glucose or pyruvate.

These results are interpreted to mean that insulin stimulates protein synthesis as measured by the transfer of labelled amino acids

from the medium into the protein of the diaphragm by a *mechanism* that is independent of a stimulation of carbohydrate metabolism It has been suggested elsewhere[10] that insulin promotes peptide synthesis by increasing the availability of energy and pentoses (for nucleic acids) required in this process The results presented here provide no support for this view Nor do they support the thesis[6]

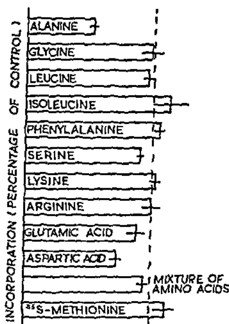


Fig 4 Effect of the addition of pyruvate to the medium on the incorporation of radioactivity from labelled amino acids into protein of the isolated rat diaphragm.
The blocks represent incorporation in the presence of pyruvate incorporation in the absence of pyruvate is shown by the dotted line
All the amino acids were the L-isomers and uniformly ^{14}C-labelled except 1 ^{14}C glycine and ^{35}S methionine

that a competition may exist between *protein* and carbohydrate metabolism for the stimulating action of insulin and that stimulation of carbohydrate metabolism by insulin might lead to a diminution of the stimulation by insulin of protein synthesis

The question remains why the behaviour of alanine is so atypical Sinex *et al* considered the possibility that in the presence of added

glucose or pyruvate the incorporation of ^{14}C alanine into diaphragm protein is diminished because of dilution of the ^{14}C alanine by unlabelled alanine formed from the added glucose and pyruvate This possibility has been tested by the addition to the medium of ^{14}C glucose or pyruvate of the same specific activity as the alanine in

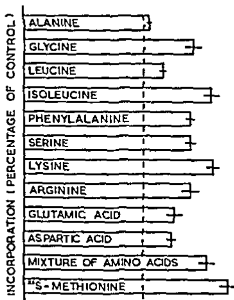


FIG 5 Effect of the addition of insulin to a medium containing glucose on the incorporation of radioactivity from labelled amino acids into protein of the isolated rat diaphragm

The blocks represent incorporation in the presence of insulin, incorporation in the absence of insulin is shown by the dotted line

All the amino acids were the L-isomers and uniformly [illegible]C-labelled except 1-^{14}C glycine and [illegible]S methionine

place of unlabelled glucose or pyruvate Under these circumstances amination of pyruvate to form alanine should not lead to a diminution of the specific activity of the added ^{14}C alanine In fact addition of ^{14}C glucose or pyruvate to the medium in addition to ^{14}C alanine leads to a greater incorporation of ^{14}C than when alanine alone is added (Fig 7) and moreover under these circumstances insulin further stimulates incorporation These results provide definite

support for the hypothesis that addition of unlabelled glucose or pyruvate depresses the incorporation of ^{14}C from ^{14}C alanine into diaphragm protein as a result of the endogenous production of unlabelled alanine[15] Direct evidence that ^{14}C-labelled amino acids are formed by diaphragm from ^{14}C glucose and pyruvate is shown in

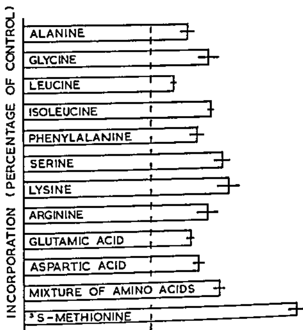


FIG 6 Effect of the addition of insulin to a medium containing pyruvate on the incorporation of radioactivity from labelled amino acids into protein of the isolated rat diaphragm.

The blocks represent incorporation in the presence of insulin incorporation in the absence of insulin is shown by the dotted line

All the amino acids were the L-isomers and uniformly ^{1} C-labelled except 1 ^{1} C glycine and ^{35}S methionine

Fig 8 A sample of protein from diaphragm incubated with ^{14}C glucose or pyruvate on hydrolysis and separation by column chromatography into its component amino acids shows peaks of radioactivity for aspartate glutamate and alanine the largest undoubtedly being alanine

It is pertinent to ask at this stage what is the effect of fasting on the ability of the isolated diaphragm to incorporate amino acids?

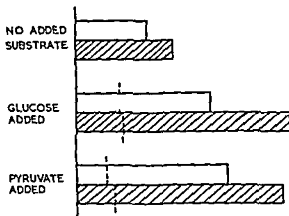


FIG 7 Eff ct of add t on of i sul ¹ C glucose and ¹C pyruvate t medium contain g C al n ne n the c po t o f C nto pr tei of the isol ted rat diaphr gm
Open blocks represent incorpor tion in the absence of i ulin h tched blocks in the *presence of in ulr*
Gl cose wa u formly C-l belled pyru te and dl-ala ine 1-¹⁴C
Broken lines ndicate the results obt ned with unl belled glucose nd pyruvate

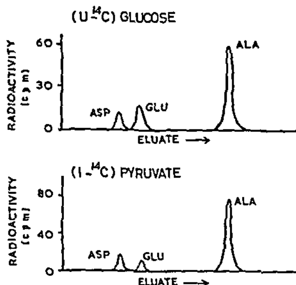


FIG. 8 Location of ¹⁴C in mi o acids from protein of isol ted r t d; phragm incubated wi h ¹⁴C glucose or pyru te
Amino acids were obtained f m diaphragm protein by acid hydrolysis and separ ted on a Dowex 50 column eluted with HCl.

Does lack of food limit the availability of energy and so depress incorporation and the ability of the diaphragm to respond to insulin? The work that has been done so far with diaphragm from 24-hour fasted rats suggests that lack of energy is not rate limiting

Fig 9 shows the results of an experiment comparing the basal incorporation and response to insulin of diaphragm from the non-fasting 24-hour and 48-hour fasting rat In fact the basal incorporation is only very slightly if at all affected by fasting and the response

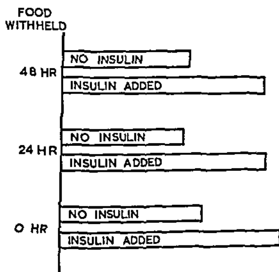


Fig 9 Effect of fasting on incorporation of ^{14}C glycine into protein of isolated rat diaphragm and its response to insulin (0 1 unit/ml)
No glucose was added to the medium

to insulin is the same in each case The response of the diaphragm and the effect of insulin on incorporation of amino acids would therefore appear to be as consistent as is the response of the diaphragm and the effect of insulin on glucose uptake

In the experiments so far discussed insulin has always been used at the rather high concentration of 0 5 unit/ml This concentration was chosen so that the amount of insulin present should not be a limiting factor However insulin at very much lower and more physiological concentrations will stimulate incorporation of ^{14}C glycine into diaphragm protein Addition to the medium of as little

as 0 5 milliunit of insulin/ml produces a half-maximal stimulation of incorporation with or without addition of glucose to the medium (Fig 10) and a smaller but still statistically significant stimulation is observed with as little as 0 05 milliunit/ml Addition of insulin to a concentration of 0 01 milliunit/ml has no observable effect Even at these concentrations there is no suggestion that addition of glucose

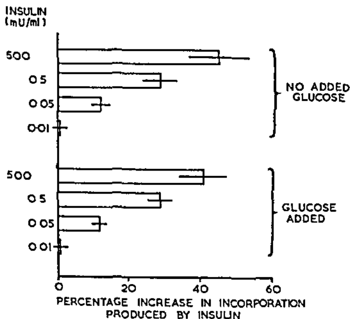


Fig 10 Comparison of th ffect of insulin at low con entrati o th incorpo t n of ^{14}C glycine into protein of the isolated rat diaph gm incubated in media w th and without dded gl cose

to the medium appreciably alters the response of the isolated diaphragm to the stimulating effect of insulin on the incorporation of ^{14}C glycine into protein

Finally administration of insulin to the normal rat stimulates the incorporation of ^{14}C glycine into the protein of the subsequently isolated diaphragm (Fig 11) whereas incorporation of ^{14}C glycine into protein of the diaphragm from the alloxan-diabetic rat is barely half that of the diaphragm from the normal rat Treatment of the

alloxan-diabetic rat with insulin raises the *ability of its isolated diaphragm to incorporated* ^{14}C glycine into its protein at nearly the *normal rate*

To summarize the results show clearly that insulin at several concentrations and under a variety of conditions stimulates the incorporation of amino acids into the protein of the rat diaphragm These experiments however do not indicate the actual mechanism by which insulin brings about this stimulation though it appears to be independent of a simultaneous augmentation of glucose utilization

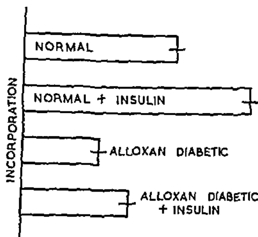


Fig 11 Effect of alloxan diabetes and of treatment with insulin of the normal and alloxan diabetic rat on the incorporation of ^{14}C glycine into protein of the isolated diaphragm
Normal rats treated with insulin received 1 unit subcutaneously 1 hour before death
Alloxan-diabetic rats received 1 unit PZ insulin daily for 4 days before death

because neither the basal incorporation nor the enhancement of incorporation by insulin is affected by the addition of glucose or pyruvate to the medium Insulin could stimulate the transfer of labelled amino acids into the protein of diaphragm in at least two ways either by enhancing the movement of amino acids from the medium into the cells of the tissue or by promoting the activity of the protein synthesizing mechanism within the cell In view of the experiments of Levine and Goldstein[12] and of Park *et al*[18,19] on the effect of insulin on the transport of sugars into muscle it seems likely that insulin enhances the entry of amino acids into the cell This

view would be in accord with the recent findings of Kipnis and Noall[8] that insulin stimulates the transfer of amino-isobutyric acid from the medium into the tissue of the whole diaphragm preparation. However the actual mechanism by which insulin brings about this occurrence still remains obscure

REFERENCES

1 Best C H (1955) *The Hypophy l G o th H rmo e Nat e d Act ons* Ed R W Sm th, O H G ebl C N H Lo g McGr w Hill Inc New Y k.
2 Forker L L. Chaikoff I L Entenman C & Tarver, H (1951) *J b l Clem* 188 37
3 Frame, E G & Russell, J A (1946) *Endocr ol gy* 39 420
4 Ingle, D J Prestrud M C & Nezamis J E (1947) *Amer J Ply ol* 150 682
5 Kerr S E. & Krikorian V H (19 9) *J b l Chem* 81 421
6 Kettteler B Randle, P J & Young F G (1957) *E gebnis e der Phys ol* 49 127
7 Kiech V C & Luck J M (1928) *J b ol Chem* 78 257
8 Kipnis D M & Noall, M W (1958) *B ch m B phys Act* 28 226
9 Krahl M E. (1953) *J b l Clem* 200 99
10 Krahl M E. (1950) *Recent Pr g Hormo e R* 12 199
11 Lawrence, R T B Salter J M & Best C H (1954) *B t med J* ii 437
12 Levine, R. & Goldstein M S (1955) *R t Pr g Horm Re* 11 343
13 Luck J M Morrison G & Wilbur L F (1928) *J b l Chem* 77 151
14 Lukens F D W & McCann S M (1955) *The Hypophy l G outh H rm e Nature and A t o* ed R W Smith O H Gaebler C N H Long M-Graw Hill Inc New York
15 Manchester K L & Young F C (1958) *B ochem J* 70 297
16 Manchester K L & Young F C (1958) *B oclem J* 70 353
17 von Mering J & Minkowski, D (1889) *A ch f xper Path Plarm* 26 371
18 Park C. R. Bornstein J & Post R L (1955) *Amer J Plysi l* 182 12
19 Park C R & Johnson L H (1955) *Amer J Phy ol* 182 17
20 Salter J M. & Best C H (1953) *Br t m d J* ii 353
21 Sinex F M MacMullen J & Hastings A B (1952) *J b ol Chem* 198 615
22 Stein W H & Moore S (1949) *Symp Q nt B l* 14 179
23 Wagner E M & Scow R O (1957) *Endocr logy* 61 419

HORMONES AND PROTEIN SYNTHESIS

A Korner

Department of Biochemistry University of Cambridge

A cell free system can be prepared from rat liver which will incorporate radioactive amino acids into protein *in vitro* (Siekevitz[19] Zamecnik and Keller[23] Sachs[16]) In its essentials the system is a simple one Rat liver is lightly homogenized at 0 in a suitable buffered medium containing salts and the unbroken cells cell debris nuclei and mitochondria are removed by centrifugation The supernatant which contains the microsomes and the soluble fraction of the cell is shaken at 37 with adenosinetriphosphate (ATP) as energy source an ATP generating system (phosphocreatine and phosphocreatine kinase were used in the experiments to be described) and guanosine triphosphate (GTP) When a radioactive amino acid is added under these conditions some of it is incorporated into protein

I have been using preparations of this type as a test system in an investigation of the way in which hormones influence the biosynthesis of proteins In doing so there is an implicit assumption that the reactions studied with this cell-free system are those which are occurring during protein biosynthesis *in vivo* No one has yet shown that net synthesis of protein occurs in this *in vitro* system but this is not surprising in view of the short life of the cell-free system Evidence is accumulating however which strongly supports the view that the reactions which occur during amino-acid incorporation into protein *in vitro* are also the reactions which occur during protein synthesis *in vivo* It is known for instance that when amino acids are incorporated into protein in this system they are located throughout the length of the peptide chain not just at its ends and the newly incorporated radioactive amino acids are joined to other amino acids by α-peptide bonds It is unlikely that the reaction studied with the *in vitro* system is an exchange between the labelled amino acid supplied and a similar unlabelled amino acid in protein because labelled amino acids once they are incorporated are not washed out of protein by incubation with excess unlabelled amino acid The view that protein synthesis is

occurring albeit at a very slow rate is also supported by the observation that more amino acid is incorporated into protein when several radioactive amino acids are added to the incubation mixture than when only one labelled amino acid with the same amount of radioactivity is present, for this suggests that several amino acids are simultaneously incorporated into protein as one would expect to occur during protein synthesis Finally Campbell *et al*[2] have

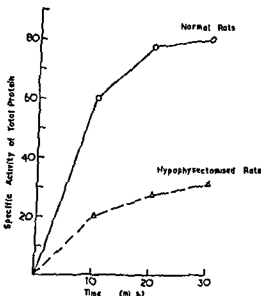


FIG 1 Specific activity of protein after various times of incorporation of DL-leucine-^{14}C in a cell free system Comparison of normal and hypophysectomized rats

shown that much of the radioactive amino acid incorporated into protein in this system goes into a specific protein namely albumin All of these observations encourage one to believe that the events observed with the *in vitro* system do reflect something of the events which occur during protein synthesis in the cell but one must of course be on one s guard to detect distorted reflections of these events

I started this work (Korner[10]) by comparing the amounts of radioactive amino acid incorporated into protein in systems of this type prepared under rigidly standardized conditions from the livers of normal rats and from rats which had been hypophysectomized

Both groups of rats ate the same ration of food Fig 1 sh amino acid is incorporated rapidly into proteins of the live paration from normal rats and that the specific activity of the p reaches a plateau after about 20 minutes A similarly shaped cu was drawn from results obtained with a preparation from the live

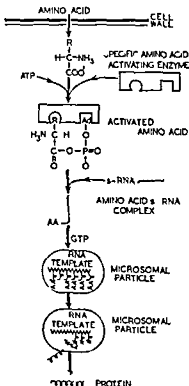


FIG 2 S hem tic representatio n f the present-day hypothe is of the sequence of re ct on wl h bring bout protein b osynthesis

of hypophysectomized rats but here the final specific activity of the protein was only about 40 per cent of that attained by the protein from the normal rats

One can now pose the question Where among the many steps which are known to occur during the biosynthesis of proteins are the pituitary hormones exerting their influence? Fig 2 summarizes

in highly schematic form, the steps which are thought to occur during protein biosynthesis Some of these steps have been quite well established on some points the evidence is not conclusive and occasionally there is frank speculation but the whole presents a reasonable working hypothesis which enables one to design experiments and which can be altered or abandoned as necessary This scheme takes no account of the protein synthesis which occurs in nuclei (Allfrey *et al*[1]) or mitochondria (Simpson *et al*[21]) since little detail is known of the sequence of reactions involved in these systems

Amino acids cross the cell wall probably by some active process (or are synthesized inside the cell) and are activated by specific amino-acid activating enzymes (Hoagland *et al*[6]) It is supposed that there are twenty enzymes of this type in the cell sap each specific for one amino acid although the existence of some of the twenty has yet to be demonstrated (Lipmann[11] Nismann *et al*[13]) The activation step requires ATP and it is suggested that an amino acyl-adenosine-monophosphate complex is formed on the surface of the activating enzyme It is possible that Vitamin B_{12} may be a co-factor of the activating enzymes (Wagle *et al*[22]) Hoagland *et al*[8] have good evidence that the activated amino acid now forms a complex with the low molecular weight (10 000–20 000) soluble ribonucleic acid (s-RNA) of the cell The amino acid is attached to the polynucleotide through adenosine and cytosine monophosphates (Hecht *et al*[5]) and it is possible that more than one subfraction of the s-RNA exists with varying affinities for each amino acid (Schweet *et al*[18]) GTP is needed in the next step which is the transfer of the amino acid from the s-RNA (Hoagland *et al*[7]) to the RNA template of the microsomal particles where it falls into a place on the template which is specific for that amino acid Crick[3] has suggested that each amino acid may be bound to a specific trinucleotide of the s-RNA and that it is this trinucleotide with the amino acid attached which enters the microsomal particle and binds to the complementary bases on the RNA template by base pairing thus placing the amino acid in a specific position It is possible to construct schemes which will satisfactorily account for the way in which the four bases of RNA taken three at a time can code without ambiguity the twenty amino acids found in proteins (Crick *et al*[4])

Littlefield *et al*[12] have produced good evidence that the actual assembly of polypeptide chains from amino acids occurs in the microsomal particles These particles are ribonucleoprotein

granules about 100–150 Å in diameter and they are probably identical with the electron dense granules noted by Palade[15] on one side of the lipoprotein membrane of the endoplasmic reticulum in rat liver and other cells When all the amino acids are present on their specific loci on the RNA template in the microsomal particles peptide bond formation occurs between them and the finished peptide with its specific amino-acid sequence peels off the template and passes first into the rest of the cell via the endoplasmic reticulum and if the cell is a secretory one into the organism as a whole

TABLE I

EFFECT OF HYPOPHYSECTOMY ON THE INCORPORATION OF LABELLED AMINO ACIDS INTO MICROSOMAL PROTEIN IN VITRO
(c p m /mg)

Soluble Fraction from	Microsomes from: Normal rat	Microsomes from: Hypophysectomized rat	
Normal rats	146	87	Ns
Hypophysectomized rats	138	76	Hs
	Nm	Hm	

One could study each of these steps separately in an attempt to identify the point or points at which pituitary hormones are exerting their effects but a short-cut is possible Centrifugation of the cell free system at 105 000 g separates the microsomes from the soluble fraction of the cell which contains the activating enzymes and the s-RNA intermediate Neither of these two parts of the cell incorporated much radioactive amino acid alone but together they incorporate as much as the unseparated system It is possible that one of these two parts of the whole system is unaffected by hypophysectomy Table I summarizes in matrix form the results of an experiment in which microsomes and soluble fractions

separated from the livers of normal and hypophysectomized rats When microsomes from normal rats (Nm) were incubated under the conditions described with soluble fraction (Ns) from normal rats the specific activity of the microsomal protein was 146c p m /mg but when microsomes (Hm) and soluble fraction (Hs) both from hypophysectomized rats were incubated together under identical conditions only about half as much amino acid was incorporated into protein If microsomes from hypophysectomized rats (Hm) are incubated with soluble fraction from normal rats (Ns) there is only a small rise in the amount of amino acid incorporated into protein compared with that obtained when the microsomes from hypophysectomized rats (Hm) were incubated with soluble fraction, also from hypophysectomized rats (Hs) Again when microsomes from normal rats (Nm) are incubated with soluble fraction from hypophysectomized rats (Hs) almost as much amino acid is incorporated into microsomal protein as when the microsomes from normal rats (Nm) are incubated with soluble fraction from normal rats (Ns) This must mean that hypophysectomy has not within the limits of these experiments resulted in a significant change in the ability of the activating enzymes to activate amino acids nor in the ability of the s-RNA to form an amino-acid complex or to transfer the amino acid from the s-RNA complex to the microsomes The lower incorporation of amino acids into protein shown by the system prepared from hypophysectomized rats compared with that from normal rats must be a result of some change in the microsomes themselves

It might be argued that microsomes from hypophysectomized rats suffer more extensive degradation during the course of an experiment than the microsomes from normal rats and that this accounts for the lower incorporation shown by microsomes from hypophysectomized rats This could be caused either by the presence of more active degradative enzymes such as cathepsins or ribonuclease in the preparation from hypophysectomized rats or by some alteration in the microsomes as a result of hypophysectomy which makes them more susceptible to these enzymic activities Experiments were therefore carried out in which the incorporation of radioactive leucine into microsomal protein was measured after various periods of pre-incubation of the microsomes either in the presence of the whole homogenate or in the presence of the soluble fraction of the cell Fig 3 shows the results obtained in one such

experiment when microsomes were pre-incubated alone and it is clear that pre-incubation does depress the ability of the microsomes to incorporate amino acids into protein but the effect of pre-incubation on microsomes from hypophysectomized rats and on those from normal rats is very similar This result supports the view that the change in the microsomes which occurs as a result of hypophysectomy is one which depresses the ability of the microsomes to incorporate amino acids in the animal and is related to the

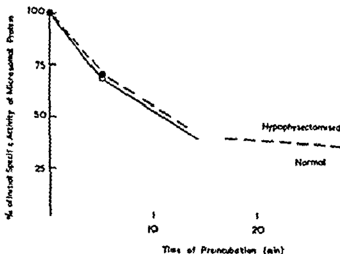


Fig 3 Effect of various times of pre-incubation of microsomes from normal and hypophysectomized rats on the specific activity of their protein after 30-minute incubation experiment. The results are expressed as percentage of the specific activity after a similar experiment shown by microsomes which had not been pre-incubated.

depression of protein synthesis in the liver which hypophysectomy (Simpson *et al*[20]) and is not a secondary which becomes apparent only as a result of the experimental techniques used

Fig 4 shows the results obtained when the microsomes separated again from the soluble fraction after incubation minutes and the radioactivity of each protein fraction determined It is clear that hypophysectomy of the significantly altered the ability of the synthesized off the RNA template of the microsomes since the

activity in the soluble fraction protein to that in the microsomal protein is similar in the preparations from normal and hypophysectomized rats

It was found that microsomes from hypophysectomiz d rats have less RNA per mg protein than microsomes from normal rats However the differences in the ability to incorporate amino acids into protein cannot be ascribed simply to a quantitative change in the RNA of the microsomes brought about by hypophysectomy for as is shown in Fig 5 a qualitative change has occurred in the

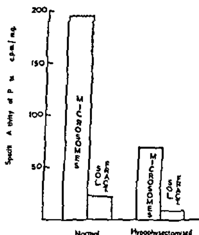


FIG 4 Specific activity of mi rosomal prote n and of soluble fraction protein from normal and hypophysectomized rat liver separated again after incubation for 30 minutes *in vitro* with L-leucine-^{1}C

RNA of the microsomes as a result of hypophysectomy The specific activity of microsomal protein is plotted against the μg of RNA present in the microsomes in Fig 5 and it is clear that the RNA of microsomes from hypophysectomized rats is less efficient on a weight for weight basis than RNA from microsomes from normal rats at incorporating amino acid into protein It is also clear from Fig 5 that addition of microsomal RNA from normal rat liver results in the maintenance of the specific activity of microsomal protein at a steady rate but a similar addition of microsomal RNA from liver of hypophysectomized rats results in a steady fall in microsomal specific activity That part of the RNA of the microsomes which is particularly concerned in protein biosynthesis is

altered as a result of hypophysectomy so that it is less capable of acting as a template When microsomes from hypophysectomized rats are added to the system one is adding partly inactive RNA

Experiments have been and are being carried out to see if treatment of the hypophysectomized rats with pituitary and other hormones can restore the ability of microsomes from hypophysectomized rats to incorporate amino acids into protein at a normal rate The results obtained with two hormones GH and insulin

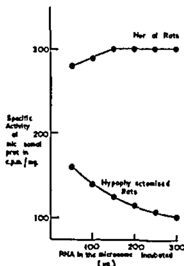


FIG 5 Specific activity of m crosom l protein from no n al a d hypophysect mized t after a 30-m n te incub t on e periment w th varyi g amounts of microsomal RNA.

will be mentioned It was found that treatment of hypophysectomized rats with ox GH was successful in markedly raising the amount of amino acid which is incorporated into protein in the *in vitro* system and it was shown that GH acts mainly by improving the incorporating ability of the microsomes and not on the reactions carried out in the soluble fraction of the cell In this respect GH is reversing the effects of hypophysectomy but full restoration of the incorporating ability of the microsomes to the normal level has not been achieved by treatment with GH alone It is probable that the

other pituitary hormones play some part in the control of the reactions which bring about protein synthesis

The anabolic action of insulin can also be demonstrated with this system Table II is a matrix diagram which shows that wh n *hypophysectomized rats are treated with insulin (0 5 units PZI/dav* for 7 days) almost three times as much amino acid is incorporated

TABLE II

EFFECT OF TREATMENT OF HYPOPHYSECTOMIZED RATS WITH INSULIN (HI) (0 5 I.U PZI/DAY FOR 7 DAYS) ON THE INCORPORATION OF LEUCINE ^{14}C INTO MICROSOMAL PROTEIN *IN VITRO*

(c p m /mg)

Soluble Fraction from	*Microsomes from* *Hypophysectomized rats*	*Insulin treated hypophysectomized rats*	
Hypophysectomized rats	25	59	Hs
Insulin-treated hypophysectomized rats	36	73	HIs
	Hm	HIm	

into protein in *in vitro* experiments compared with that incorporated into the protein from untreated hypophysectomized rats In this case not only were the microsomes from insulin-treated rats able to incorporate more activated amino acids than those from untreated rats but in addition the soluble fraction of the cell was better able to prepare the radioactive amino acid for incorporation This is shown by the fact that incubation of the microsomes from hypophysectomized rats (H) with soluble fraction from insulin treated hypophysectomized rats (HI) resulted in greater incorporation of amino acid than occurred when the microsomes and soluble fraction were both from untreated hypophysectomized rats

Table III summarizes the results of preliminary experiments with rats made diabetic with alloxan The lower incorporation of amino

acids into protein shown by the alloxan-diabetic rats (D) compared with control rats (N) is somewhat restored when the diabetic rats were treated with insulin (DI) but not altered significantly when they are treated with ox GH (DG) These observations lend support to the view (see Ketterer *et al*[9] for discussion of this subject) that GH requires the presence of insulin before its anabolic action can be fully realized but that insulin can cause anabolism in the absence of GH (Salter and Best[17])

TABLE III

EFFECT OF ALLOXAN DIABETES (D) AND OF TREATMENT OF ALLOXAN-DIABETIC RATS WITH INSULIN (2 I.U PZI/DAY FOR 5 DAYS) (DI) OR GROWTH HORMONE (0 2 MG /DAY FOR 5 DAYS) (DG) ON THE INCORPORATION OF LEUCINE C INTO MICROSOMAL PROTEIN *IN VITRO*

(c p m./mg)

Soluble Fraction from	*Micr omes f om* Normal ats	Diabet c rats	Insul n-tre ted d abetic rats	GH-tr t d d abetic ts	
N r l ats	95	77	89	74	Ns
Diabet c r t	79	66			Ds
Insul n-treated di bet c rats	87		89		DIs
GH-treated d bet c r t	81			72	DG
	N 1	Dm	DIm	DGm	

Finally I want to mention the results of some experiments on the effect of hormones on the incorporation of amino acids into protein *in vivo* which are in agreement with the conclusions drawn from the *in vitro* experiments which have been discussed In these experiments amino acids were injected into the femoral vein of the rats and successive lobes of the liver removed . 10 and 20 minutes after the injection Each lobe of liver was homogenized and the soluble fraction and microsomes separated from one microsomes were treated with sodium deoxycholate ir described by Littlefield *et al*[12] and separated into two microsomal particles containing about 50 per cent RN

cent protein (Palade and Siekevitz[15]) and the fraction probably consisting of the lipoprotein endc culum The radioactivity of the proteins of each of fractions was assessed and are plotted in Fig 6 which › amino acid is rapidly incorporated into protein in the particles and that the radioactive protein is transferred deoxycholate soluble fraction of the microsomes and ‹ soluble fraction of the cell (cf Littlefield *et al*[12])

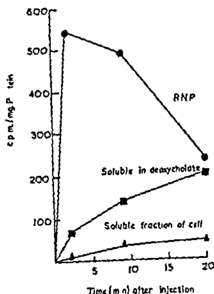


FIG 6 Specific activity of protein of microsomal particles (–●–) fraction soluble in sodi deoxycholate (–■–) and the soluble fraction of the cell (–▲–) at various times after i venous injection of radioactive amino acid into a normal rat.

In hypophysectomized rats less amino acid is incorporated ‹ protein in the microsomal particles than in normal rats and in sequence less radioactive protein is transferred to the deoxycholate soluble fraction of the microsomes and to the soluble fraction of the cell Treatment of the hypophysectomized rats with ox GH or with insulin increased the incorporation of amino acids into protein in the microsomal particles (Fig 7) and consequently increased the specific activity of the other two factions

SUMMARY

Hypophysectomy reduces the amount of amino acid which is incorporated into the proteins of liver *in vivo* or *in vitro* The activity of the amino-acid activating enzymes is not significantly altered by hypophysectomy nor is the ability of the s-RNA to form an amino-acid-s-RNA complex The microsomes themselves are

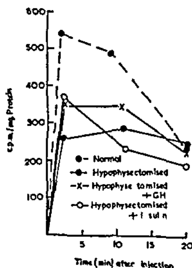


Fig 7 Specific act ity of m crosomal part cle protein from normal, hypophysectomized GH-treated hypophysectom zed and insulin-tre ted hypophysecto zed rats t variou t mes fter intr enous injection f rad oactive amino acid

less able to incorporate amino acids into protein as a result of hypophysectomy probably because of a change in the RNA of the template Treatment of hypophysectomized rats with ox-growth hormone or with insulin could restore in part the impaired ability of the microsomes to incorporate amino acids into protein Alloxan-diabetic rats showed a similar impaired amino-acid incorporating ability which could be restored by treatment of the diabetic rats with insulin but not under the conditions described by treatment with ox-growth hormone

Acknowledgments

I wish to thank the Council of the Royal Society for a Grant in aid Miss M B Thomas who performed the hypophysectomies and Mr D C Gardiner for highly skilled assistance It is a pleasure to thank Professor F G Young F R S for his interest in this work and for his encouragement

References

1 Allfrey V G Mirsky A E & O awa S (1957) *The Chem cal Basis of Hered ty* Ed W B McElroy and B Glass Johns Hopkins Press Baltimore
2 Campbell P N Greengard O & Kernot B A (1958) *Biochem J* 68 18
3 Crick F H C (1958) *Soc Exp Biol Symp* 12 London
4 Crick F H C Griffith J S & Orgel, L E (1957) *Proc Nat Acad Sci* 43 416
5 Hecht L I Stephenson M L & Zamecnik P C (1958) *Fed Proc* 17 944
6 Hoagland M B Keller E B & Zamecnik P C (1956) *J b ol Chem* 218 345
7 Hoagland M B Stephenson M L Scott J F Hecht L I & Zamecnik, P C (1958) *J biol Chem* 231 241
8 Hoagland M B Zamecnik, P C & Stephenson M L (1957) *B o him bi phys A ta* 24 215
9 Ketterer B Randle P J & Young F G (1957) *Ergeb Phys ol* 49 127
10 Korner A (1957) *Nature* 181 42
11 Lipmann F (1958) *Proc Nat Acad Sc* 44 67
12 Littlefield J W Keller E B Gross J & Zamecnik P C (1955) *J b ol Chem* 217 111
13 Nismann B Bergmann F H & Berg P (1957) *Biochim biophys Acta* 26 639
14 Palade, G E (1956) *J B ochem B opl ys Cytol* 2 85
15 Palade, G E & Siekevitz P (1956) *J B ochem Biophys Cytol* 2 171
16 Sachs H (1957) *J biol Chem* 228 23
17 Salter J M & Best C H (1953) *Br t med J* II 353
18 Schweet R S Bovard F C Allen E & Glassman E (1958) *Proc Nat A ad Sci* 44 173
19 Siekevitz P (1952) *J biol Chem* 195 549
20 Simpson M E Evan H M & Li C H (1949) *Growth* 13 151
21 Simpson M V Bates H M & Craddock V M (1958) *Fed Proc* 17 1232
22 Wagle, S R Mehta R & Johnson B C (1958) *Fed Proc* 17 1305
23 Zamecnik P C & Keller E B (1954) *J b ol Chem* 209 337

DISCUSSION

KRAHL For some years it has been clear that insulin has both short term nd long term effects on protein synthesis In these two extremely lucid papers from Professor Young s laboratory both aspects of the effect of nsulin on protein synthesis have been discussed The second paper by Dr Korner opens up new avenues of experiment regarding the long-erm effects of insulin on protein synthetic processes The remainder of ny discussion will be devoted to describing some experiments of our own which cover somewhat the same ground as those discussed by Dr Man hester with a slightly different point of view

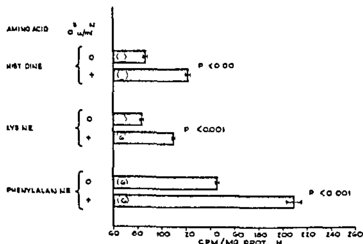


FIG 1 I corporation of C[1] Amino acids into muscle protein
Effect of insulin in the absence of gl cose

My own interest in this as pointed out by Dr Manchester began in 195- when Sinex, Hastings and MacMullen on the one hand and I on the other independently observed and reported that insulin apparently had an effect to stimulate amino-acid incorporation into protein in the absence of added extracellular glucose This effect of insulin, which is independent of that on glucose transport, apparently represents one facet of the general action of insulin to initiate cellular rearrangements favouring anabolism (Krahl *Perspectives Biol and Med* 1957 1 69) Two years ago my colleague Dr Wool began further experiments in this field to establish the reality of this effect of insulin in the absence of glucose his results have been remarkably similar to those described by Manchester I will review them rapidly to show that in one field, at any rate two

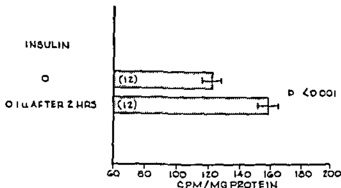


Fig 2 Incorporation of C^{14} Histidine into muscle protein Effect of insulin added after 2 hours prior incubation — no glucose present

independent groups of investigators have got exactly the same or nearly the same results This is extremely rare in anything having to do with the mechanism of insulin action

In diaphragms from *f d* rats insulin stimulates incorporation of histidine lysine or phenylalanine into protein *even in the absence of added glucose*

In an effort to reduce the amount of glucose which might have been

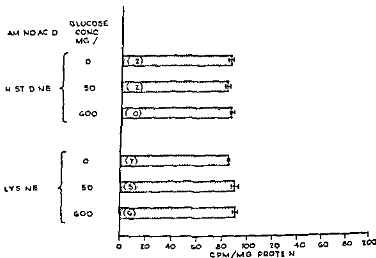


Fig 3 Incorporation of C^{14} Amino acids into muscle protein Effect of glucose concentration

carried over by the diaphragms from the fed rats we incubated the diaphragms for ‿ hours without glucose or insulin then added insulin to half the samples and continued the incubation for ‿ hours more Under these conditions in which any glucose in the extracellular fluid would be exhausted we still observed a stimulation by insulin of amino-acid incorporation

These are further control experiments with diaphragms from *fed* rats The argument was that if the effects of insulin described above were due to increased use of glucose under the stimulus of insulin then addition of enough glucose would produce the same effect as insulin it had been

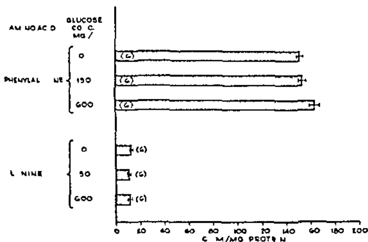


FIG 4 I o poration of C A i o acid into muscle protein. Effect of gl cose concentr tion

previously shown by Gemmill years ago and by us under the present experimental conditions, that increase in glucose concentration led to an increase in glucose use However as you see the addition to the medium of 150 mg per cent or even 600 mg per cent glucose did not promote incorporation of histidine lysine phenylalanine or alanine into protein of diaphragms from *f d* rats We therefore concluded that the effect of insulin shown in Figs 1 and 2 was in leed one concerned with amino acids *per se* and not with glucose transport

The original experiments undertaken by Wool were concerned simply with establishing an effect of insulin on amino-acid incorporation in the absence of glucose By the time this had been completed other ques ions

arose what is the mechanism of this effect of insulin in the absence of glucose? In particular is this effect of insulin completely accounted for by an effect on the transport or accumulation of amino acids? The present answer to this second question appears to be negative for reasons stated below

Kipnis and Noall (*Biochim Biophys Acta* 1958 28 226) working at Washington University reported that insulin increased the rate of accumulation of a non utilizable amino acid α aminoisobutyric acid (AIB) by diaphragms *in vitro* There are differences between the effect of insulin on AIB accumulation and on amino-acid incorporation into protein (Fig 5)

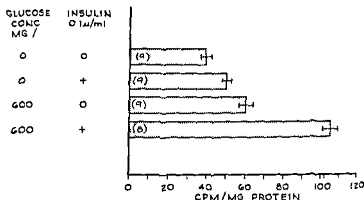


FIG 5 Incorporation of C^{14} Histidine into muscle protein
Effect of glucose and insulin 48 hours fasted rats

First insulin promotes AIB accumulation but not significantly the incorporation of amino acid into protein by diaphragms from animals fasted for 2 or 3 days This demonstrates clearly that an increase in radioactivity of the protein does not necessarily follow an increase in the *specific radioactivity of the amino acids of the intracellular pool* Secondly with diaphragms from *fasting* animals addition of glucose and insulin to the incubation medium gives no greater stimulation of AIB accumulation than insulin alone in contrast glucose and insulin act synergistically upon incorporation of amino acid into protein giving a stimulation larger than the sum of the stimuli produced by either alone From these facts Wool and I have reached the working hypothesis that insulin may *have some effect on peptide synthesis in addition to that on amino-acid* transport

In summary insulin promotes peptide synthesis in rat ... mind Two components of the insulin effect can be ... diaphragms from fed rats insulin stimulates incorporation of amino ... into protein even in absence of added glucose Whether this is accou... by an insulin effect on amino-acid transport from extracellular to cellular phase remains to be settled by further experiment In diaph... from 48 hour fasting rats insulin alone has no significant effect on ... synthesis glucose alone promotes it, and insulin and glucose tog... synergistically A similar situation obtains for synthesis of ... or protein in liver slices from mildly diabetic rats This component insulin anabolic action is apparently dependent on stimulation of g... utilization

In conclusion, I should like to mention that Professor H Cl... and co-workers have shown that other hormones do have effects ... acid accumulation by animal tissues For example after injection, adrenal C 11 oxysteroids promote accumulation of AIB by liver but ... by certain other tissues this may be related to the conversion of protein to glycogen Also oestrone promotes accumulation of AIB by uterus but not by other tissues such as liver upon which oestrone has no particular growth-promoting effects So we are now in the position of having to unravel the contributions of individual hormones by way of amino-acid transport and to show whether or not these various hormones have still other effects on protein synthesis

DICKENS Thank you very much Professor Krahl for your interesting criticism and confirmation of some of the work of the preceding speakers It is obvious to us all now I think, that there is room for alternative hypotheses on the action of insulin on the incorporation in amino acids as in most other fields concerning insulin action The balance of evidence seems to be in favour of direct stimulatory effect of insulin on amino-acid incorporation which is apparently independent of glucose transport, as we find Professor Krahl and Dr Manchester in agreement on this basic point

There is also the question of the extent of energy required for the protein synthesis which bears upon the work of Dr Korner who has in a very original way I think applied the findings of Lipmann and Zamecnik and others on modern views of protein biosynthesis to try to localize the exact point of action and if I understood it rightly tends to pin this down to an alteration in the effectiveness of ribonucleic acid in promoting protein incorporation in the cell.

So we have had at least two views which are perhaps not easy to reconcile but in these matters there is more than one factor affected, and this is no new situation

PETERSON May I make two little points The first is did Dr Korner

arose what is the mechanism of this effect of insulin in the absence of glucose? In particular is this effect of insulin completely accounted fo by an effect on the transport or accumulation of amino acids? The present answer to this second question appears to be negative for reasons stated below

Kipnis and Noall (*Biochim Biophys Acta* 1958 28 226) working at Washington University reported that insulin increased the rate of accumulation of a non-utilizable amino acid α-aminoisobutyric acid (AIB) by diaphragms *in vitro* There are differences between the effect of insulin on AIB accumulation and on amino-acid incorporation into protein (Fig 5)

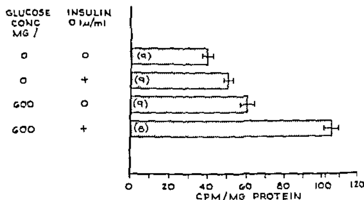


FIG 5 Incorporation of C^{14} Histidine into muscle protein
Effect of glucose and insulin 48 hours fasted rats

First insulin promotes AIB accumulation but not significantly the incorporation of amino acid into protein by diaphragms from animals fasted for 2 or 3 days This demonstrates clearly that an increase in radioactivity of the protein does not necessarily follow an increase in the specific radioactivity of the amino acids of the intracellular pool Secondly with diaphragms from *fasting* animals addition of glucose and insulin to the incubation medium gives no greater stimulation of AIB accumulation than insulin alone in contrast glucose and insulin act synergistically upon incorporation of amino acid into protein giving a stimulation larger than the sum of the stimuli produced by either alone From these facts Wool and I have reached the working hypothesis that insulin may have some effect on peptide synthesis in addition to that on amino-acid transport

In summary insulin promotes peptide synthesis in rat diaphragm muscle Two components of the insulin effect can be identified In diaphragms from *fed* rats insulin stimulates incorporation of amino acids into protein *even in absence of added glucose* Whether this is accounted for by an insulin effect on amino-acid transport from extracellular to intra cellular phase remains to be settled by further experiment In diaphragms from 48 hour fasting rats insulin alone has no significant effect on peptide synthesis glucose alone promotes it and insulin and glucose together act synergistically *A similar situation obtains for synthesis of glutathione* or protein in liver slices from mildly diabetic rats This component of insulin anabolic action is apparently dependent on stimulation of glucose utilization

In conclusion, I should like to mention that Professor H Christensen and co-workers have shown that other hormones do have effects on amino-acid accumulation by animal tissues For example after injection the adrenal C-11 oxysteroids promote accumulation of AIB by liver but not by certain other tissues this may be related to the conversion of protein to glycogen Also oestrone promotes accumulation of AIB by uterus but not by other tissues such as liver upon which oestrone has no particular growth-promoting effects So we are now in the position of having to unravel the contributions of individual hormones by way of amino-acid transport and to show whether or not these various hormones have still other effects on protein synthesis

Dickens Thank you very much Professor Krahl for your interesting criticism and confirmation of some of the work of the preceding speakers It is obvious to us all now I think that there is room for alternative hypotheses on the action of insulin on the incorporation in amino acids as in most other fields concerning insulin action The balance of evidence seems to be in favour of direct stimulatory effect of insulin on amino-acid incorporation which is apparently independent of glucose transport as we find Professor Krahl and Dr Manchester in agreement on this basic point

There is also the question of the extent of energy required for the protein synthesis which bears upon the work of Dr Korner who has in a very original way I think applied the findings of Lipmann and Zamecnik and others on modern views of protein biosynthesis to try to localize the exact point of action and if I understood it rightly tends to pin this down to an alteration in the effectiveness of ribonucleic acid in promoting protein incorporation in the cell

So we have had at least two views which are perhaps not easy to reconcile but in these matters there is more than one factor affected and thi is no new situation

Petrow May I make two little points The first is did Dr Korner

use rats that were adult? Because as hypophysectomy alters the pattern I wondered whether castration would alter the pattern There may after all be some sort of synergistic effect between the testosterone type of compound and insulin and that brings me to the next point

It has been suggested recently — I think in the *B M J* within the last 2 years — that insulin is the *major anabolic hormone of the body* Now that may well be true and I think probably prior to puberty it is difficult to see where the anabolic effect is going to come from There is no testosterone present as far as we know in the tissues But after puberty you have I think a synergistic effect between androgens and the insulin hormone

KORNER In answer to the specific points raised by Dr Petrow — (*a*) The rats used in the experiment I have described were young adults i e 3 5 months of age and weighing 150-200 gms (*b*) Preliminary experiments with castrated male rats indicate that androgens are concerned in the amino-acid incorporation into protein in the system I have described

YOUNG I did not understand clearly whether Dr Petrow was suggesting that the growth hormone of the pituitary gland was not an important anabolic hormone of the body I understood him to say that insulin was the major one Did I understand correctly?

PETROW That is the suggestion put forward in this recent paper

YOUNG I do not think that we can dismiss the effect of the pituitary gland on growth as lightly as that I myself am very impressed by the importance of the pancreas in stimulating anabolism of protein but the work of Scow in recent years has shown that in the rat at any rate one sees an anabolic effect of growth hormone in completely depancreatized animals maintained on a constant dosage of insulin While one should not underrate the importance of insulin in anabolic processes such as protein synthesis I think one also should not be so much swayed by such ideas as to demote the pituitary gland in general and its growth hormone in particular

LONG I would like to address a question to Dr Korner Since removal of the hypophysis removes two types of effects on protein metabolism the growth hormone as well as the adrenal corticotrophic hormone has he studied these effects in animals deprived of their adrenals or previously treated with large amounts of ACTH or hydrocortisone? One might anticipate rather opposite effects with ACTH and growth hormone

KORNER Thank you Professor Long for your question on the role of the adrenals in this amino-acid incorporating system My preliminary observations show that small doses of ACTH given to normal or hypophysectomized rats decrease the amino-acid incorporation into protein obtained with the microsomal system but that when small amounts of ACTH are given to hypophysectomized rats together with GH a some-

what greater incorporation is seen than when GH is given alone I may add that adrenalectomy results in decreased incorporation of amino acids into protein *in vitro* and that the effect is exerted both on the microsomal and on the soluble fraction of the cell

DALE I am not at all sure that my intervention is properly relevant to the brilliant contributions to this symposium which we are supposed now to be discussing and which have been concerned with the evidence for the promotion by insulin of the synthesis of cell proteins from amino acids and with the more detailed method of that synthesis Listening to these communications however and more especially to some of the earlier and equally important ones dealing with the effect of insulin on the relation between the glucose contents of the blood as it enters and as it leaves the liver in the living animal I have been wondering whether in discussing the significance of the positive and negative balance observed sufficient attention has in all cases been given to both sides of the account Most of you will be aware that I have never had any proper ground for a claim to be regarded as a carbohydrate biochemist or indeed as a real biochemist of any kind I cannot help recalling however the experiences which I had in the very early days of research on insulin and its actions in as happy and lively a collaboration as it was ever my privilege to enjoy with Charles Best whom we would so gladly have seen here today Joseph P Hoet who is actually here and my own colleague at that time the late H P Marks Students of the effects of insulin were even then faced with what seemed to be the paradox that insulin as was to be expected caused glucose to disappear from the circulatory blood, but that nobody had been able to discover what became of it The first and natural supposition had been that it would have been synthesized to glycogen and deposited as such in the tissues When however an excessive dose of insulin was injected into normal mice with the result that they fell into hypoglycaemic coma in some 20 to 30 minutes so far from the glucose which disappeared from the blood being discoverable anywhere in the body as extra glycogen all the naturally occurring glycogen of the liver muscles or any other tissue had also disappeared leaving an animal practically devoid of free carbohydrate in any recognizable form The theory which was then rather freely promulgated indeed was that insulin caused the transformation of carbohydrate into some unknown form in which it escaped detection by available methods With my then still young collaborators however I found myself looking at the apparent paradox from a different angle We reckoned that the amount of free carbohydrate in any form which was available at any one moment in the organism of a small animal such as a mouse would by itself only suffice to meet the needs of its rapid oxidative metabolism for say half an hour or so Normally the balance was being constantly maintained

by the catabolism of cell proteins with a resulting formation of nitrogenous excreta and glucose which would provide for a long maintenance of the blood sugar even in the absence of food

We thought therefore that the paradoxical effect of insulin could be accounted for if we could suppose that apart from an initial promotion of the synthesis of blood sugar into cell glycogen it would also effectively prevent the production of new glucose from protein The oxidative metabolism would then be essentially concentrated on the small quantity of carbohydrate available as such at the time of injection and the prevention of new formation from protein would under such conditions become the central feature of the insulin effect and lead to an early collapse and death from carbohydrate exhaustion The only experimental contribution which we were able to make was to show that in the eviscerated preparation with blood sugar maintained by artificial infusion all the extra sugar disappearing from the blood under insulin could be accounted for by glycogen deposition in the muscles and by oxidation. We had no means available for testing our supposition that insulin would also stop or restrict the new formation of sugar from protein in the liver I must leave it to our colleagues who have just given us such convincing evidence of the promotion by insulin of synthesis of proteins from amino acids to judge whether the prevention of protein breakdown which we envisaged would not be another aspect of the synthetic action which they have demonstrated

My only excuse for speaking is that I have had the impression – very probably a mistaken one – that some of the results under discussion earlier today have appeared once again to be producing paradoxical problems of the carbohydrate balance sheet and I have wondered whether these may again have involved a neglect of the factor of the suppression of the new formation of carbohydrate from protein which I still suspect of being perhaps the most important of the actions of insulin I can only thank you all for tolerating what may be an irrelevant intervention, and for humouring an old man s anecdotage!

DICKENS There is no need for me to say what a pleasure it was to hear such a clear and vigorous account from Sir Henry who has put the whole thing in proportion by his remarks We are very glad that he had this accident which meant his entry into this work at that time

CORI I think of course Sir Henry that new formation of carbohydrate is a process which still exists The debate was really whether this new formation is from protein only or whether fatty acids are involved in this process as well During the period which I mentioned earlier when diabetes was explained by over-production then this could only be done by assuming that the fatty acids were giving a net gain in carbohydrates but since we know something about intermediary metabolism of fatty

acids and proteins the present idea is that fatty acids are not important contributors of carbohydrate although labelled fatty acids through a common carbon pool may seemingly show that fatty acids go to carbohydrates So protein is at the present time supposed to be the main source of the formation of new carbohydrate and it is possible that if insulin promotes the synthesis of protein that this means simultaneously a decreased breakdown of protein In this sense your point is very well taken that this might very well result in a lessening of new formation of carbohydrate simply by shifting the equilibrium from protein breakdown to one of protein synthesis

DALE Do you not think that possibility does come into some of the problems that we were discussing earlier today?

CORI Yes I should think so very much especially even in diabetes where protein catabolism is increased this action of insulin may well play an important role

PART VI

BIO-ASSAY OF INSULIN

Chairman PROFESSOR T RUSSELL FRASER





SUGGESTED IMPORTANCE OF ADIPOSE TISSUE AS A SITE OF INSULIN ACTION AND AS A MAJOR SITE OF METABOLIC INTERRELATIONS BETWEEN CARBOHYDRATES AND FATS*

ALBERT E RENOLD ALBERT I WINEGRAD BERNARD JEANRENAUD AND DONALD B MARTIN

The Baker Clinic Research Laboratory and the Departments of Medicine Harvard Medical School and the Peter Bent Brigham Hospital Boston Massachusetts

When considering the metabolic interrelations between carbohydrates and fats it is customary to be primarily concerned with the liver I should like to review observations which suggest that our concern should be equally devoted to adipose tissue a tissue highly specialized for the synthesis of fatty acids from glucose as well as from other precursors With relation to glucose the significance of this problem may perhaps be underlined by recalling that as first definitely pointed out by Stetten and Boxer the storage of glucose carbon as fatty acids easily equals and frequently exceeds its storage as glycogen

Although most of the observations to be reported will concern the effects of insulin upon adipose tissue I should like to make it clear at the outset that they will not really bear upon the *mechanism* of insulin action In this tissue we are as yet at the stage of describing *what* insulin does rather than *how* it does it

The studies to be presented as well as our general interest in adipose tissue and its hormonal control have been early and lastingly influenced by the extensive work carried out in the laboratories of Wertheimer and Shapiro[17 18 20] in Jerusalem Hausberger in Philadelphia[8 13] and Favarger in Geneva[5 6] Wertheimer s classic review published in 1948[20] emphasized the observation that glycogen accumulates in the cytoplasmic rim of adipose tissue cells under certain rather specific circumstances among which the administration of insulin to diabetic rats was prominent When first

* S pp ted in part by th Adl F und t n In Rye N w Yo k d the Nat nal In titut f Health, United States P bl c Health S rvi e B th sd Ma yl nd (G t No A 640)

concerned with this tissue in 1949[15] we observed (Fig insulin was injected into the groin fat pad of six dia' being injected as control on the other side) glycogen d t greater in the insulin-injected pad suggesting a *direct*, l insulin upon this tissue The glycogen levels shown t become quite considerable when related to fat-free tissu

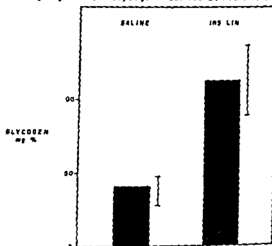


Fig 1 Local action of insulin in six diabetic rats. Data from Renold *et al.*[15]

Subsequently the existence of a direct effect of insulin upon pose tissue suggested here was established *in vitro* by Krahl[10] by Haugaard and Marsh[7] using glucose uptake and oxygen sumption respectively as parameters of insulin effectiveness T insulin effect upon sliced brown adipose tissue has also' as pointed out yesterday by Chain but this tissue of almost g' appearance should probably not be considered as representative adipose tissue in general

Having paid prolonged and respectful attention to the liver the intervening years we have recently renewed our interest in adipose tissue using the epididymal fat pad of the male rat as tissue preparation carefully avoiding tearing, slicing chilling or any un necessary handling in the process of securing it This tissue is really

better termed a fat sheet than a fat pad since it pending medium and in the appropriate size rat is ... ½ to at most 1 millimetre thick in any one point out to those justly critical of the unhomogen- existing in isolated ti sues that in this instance glycog *in vitro* may be shown to proceed rather homogeneo

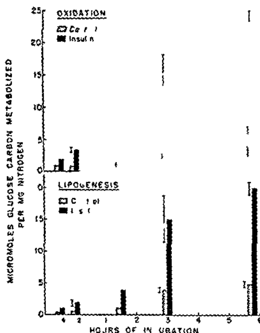


FIG 2 Effects of insulin added *in vitro* on oxidation of gluc e-U-^{14}C to CO and on corporation of glucose carbon into lip de by rat adipose tissue after incub tion pe varying from 15 minutes to 6 hours In ubation carried out in Kreb bicarbonate ontaining o mM/l of glucose. Insulin concentratio when present 0 1 unit per ml

out (unpublished observations of Blacklow and Martin) The tissi is symm trical and furthermore may be quite conveniently subdivided into as many as three pieces for each pad a total of six pieces for each animal Early results concerning insulin effects obtained under these conditions revealed them to be of unexpected magnitude and the decision was made to engage in a rather long-term

programme of re-evaluation of the effectiveness of insulin and other hormones upon this tissue Some of the observations gathered since[1, 16-1] will be summarized here

Fig 2 illustrates the effects of insulin added *in vitro* at rather high concentration (0 1 unit/ml) upon the oxidation of glucose carbon (using uniformly labelled glucose) to CO_2 and its utilization for the synthesis of long-chain fatty acids It is apparent that the effect of insulin is *marked early* (15 minutes) and *persistent* (up to 6 hours) although some diminution of effectiveness during the last 3 hours is suggested I should like to point out that on the balance sheet these two metabolic fates of glucose carbon (i e oxidation and lipo genesis) accounted for approximately two thirds of the glucose disappearing from the medium both with and without added insulin Also that it is not necessary to use labelled glucose if adipose tissue is incubated in a Warburg flask without centre well in the *presence of glucose the respiratory quotient is initially close to* unity and net gas formation or uptake is therefore minimal *If* insulin is now tipped in from the side-arm the respiratory quotient increases immediately to between 2 and 3 and net gas formation becomes evident proceeding in linear fashion from within *seconds* of insulin addition to at least 3 hours later Observations in this system are presently actively being pursued in collaboration with Dr Eric G Ball in the Department of Biological Chemistry Harvard Medical School *

Insulin effects upon adipose tissue occur at remarkably low concentrations of the hormone as shown in Fig 3 In this instance six *pieces of adipose tissue from one rat* were used and a clear-cut insulin effect was observed at the concentration of 10 micro-units per ml (or 10^{-5} units per ml) a quite consistent and uniform finding whatever the index of insulin activity used Still lower concentrations are frequently but not invariably effective Parenthetically I might recall that the most generally accepted figure for insulin like activity of normal human plasma obtained in the *fasting state* is of the order of 100 micro-units per ml

Although effects of insulin added *in vitro* upon fatty acid synthesis have been described in isolated liver these effects could *not* be obtained in liver isolated from diabetic animals [2,4] It was of interest to find therefore that the direct effect of insulin upon glucose oxida-

* Some of these results have been published by Ball E G Martin D B and Cooper O (1959) *J biol Chem* 234 774

tion and lipogenesis from glucose could be clearly dem adipose tissue obtained from diabetic animals as well alth the baseline activity of the diabetic tissue and its insulin was considerably less than normal (Fig 4 second pair of col Fig 4 further demonstrates that when the insulin effect upon genesis was tested in the presence of a variety of substrates specifically here in the presence of labelled glucose or acetate

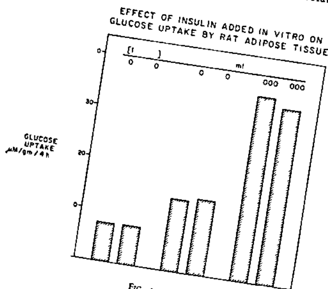


FIG 3

and of labelled acetate with in addition unlabelled glucose) it became clearly apparent that a stimulation of lipogenesis in the presence of insulin required the presence of glucose in the medium This strongly suggests that accelerated glucose metabolism is a prerequisite for insulin-induced accelerated lipogenesis This is of course in excellent agreement with present concepts concerning cofactor requirements for the synthesis of fatty acids concepts entirely based upon observations derived from liver preparations 11 19

In adipose tissue these concepts were further tested by comparing the effects of insulin upon the metabolism of the first and last carbons of glucose From consideration of the figures summarized in

Table I it is apparent that (as previously shown by Milstein[14] carbon dioxide only) unstimulated adipose tissue metabolizes carbon-1 than carbon-6 to CO and more carbon-6 than or to fatty acids suggesting the likely presence of the phosphogl oxidative pathway in this tissue In confirmation I have authorized to state that Drs Shaw and Winegrad working in

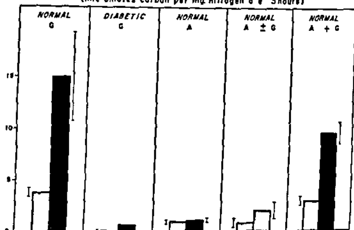


FIG 4 Each column refers to substrate carbon incorpor ted into adipose tissue fatty acids during the period of incubation the black column being the mean activity of the tissues in the presence of insulin (0 1 unit per ml) the shaded column representing the mean of the paired controls. Normal and Diabetic refers to the state of the animals from which the tissues were obtained. The nature of the substrate is indicated by the letters at the top of each pair of columns as follows G* glucose-U [14]C, 20 mM/l A* acetate-1-[14]C 60 mM/l G unl belled glucose 10 mM/l Note that in the fourth pair of columns no insulin was added and that the variable examined in this instance is the addition of unlabelled glucose The bracket adjoining each column indicates the standard error of the mean of the whole group and does not make allowance for the paired nature of the d ta

Stadie s laboratory have demonstrated the presence of considerable activity of both glucose-6-phosphate and 6-phosphogluconate dehydrogenases in this tissue It is also apparent from the table that when insulin is present both metabolic fates of both carbons are activated although not to a similar extent in all instances I should like to concentrate first on the fatty acid data and point out that the ratio of carbon-1 to carbon-6 incorporated into fatty acids remains the same albeit at a much higher level of activity The ratio of CO_2

from carbon-1 to CO_2 from carbon-6 however is severely shifted towards carbon-1 It is customary to dispose of such discrepancies by pointing out the relative lack of reliability of any calculations based upon CO_2 data alone (reflecting perhaps variations in pool sizes for various paths from substrate to CO_2) although this reasoning

TABLE I

EFFECTS OF INSULIN ADDED IN VITRO ON THE METABOLISM OF GLUCOSE-1-^{14}C BY RAT ADIPOSE TISSUE*

Number of experiments	Insulin	Oxidation to CO_2		Incorporation into fatty acids	
		carbon-1	carbon-6	carbon-1	carbon-6
6	0	0 92	0 24	0 19	0 38
6	+	6 36	0 33	3 06	6 31

* All values expressed as micromoles of glucose carbon-1 or carbon-6 per mg tissue nitrogen. Incubation carried out for 3 hours in Krebs bicarbonate buffer containing 20 millimoles per litre of glucose Insulin concentration when present 0 1 unit per ml

does not appear entirely convincing in this instance Be that as it may however the more standard and rather generally accepted mode of calculating relative activities of the glycolytic and the phosphogluconate-oxidative pathways (based on fatty acid data) demonstrates a completely similar distribution of glucose-6-phosphate metabolism between the two pathways with and without insulin (Table II) *We attach importance to this mainly because it* clearly suggests that the major action of insulin upon this tissue becomes effective *before glucose-6-phosphate* leaving increased transport and/or phosphorylation as the only known determinants Which we could not say

Parenthetically I should like to add that we have utilized the thus observed highly active CO_2 formation from glucose-1-^{14}C as a likely and in our hands the most appropriate indicator of insulin-like activity for bio-assay purposes [14] The sensitivity of this bio-assay system is 10 micro units per ml (13- ± 4 per cent of control value 4 being the standard error for twenty observations) This insulin sensitivity is not altered by adrenalectomy or by cortisone overdosage [9] *a finding in agreement with the observations of Levine in* the eviscerated dog discussed elsewhere in this symposium Further-

more the depressed lipogenesis from pyruvate exhibited by adipose tissue from alloxan-diabetic rats is not returned toward normal by superimposed adrenalectomy [3] in marked contrast to *hepatic* lipogenesis from pyruvate under these conditions [4] This discrepancy could be established even in the same group of animals as illustrated in Fig 5 and is presently interpreted as suggesting that the adrenal

TABLE II

EFFECT OF INSULIN UPON THE METABOLISM OF CARBON-6 OF GLUCOSE TO FATTY ACIDS BY WAY OF EMBDEN MEYERHOF OR ALTERNATE PATHWAYS*
(Calculation discus ed n Reference 21)

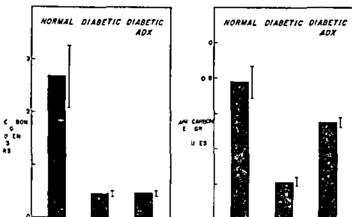
Animal no	*Total*	*Via Embden Meyerhof pathway*	*Via alternate pathway*	*Via alternate pathway (per cent of total)*
		No insulin added		
116	0 37	0 24	0 13	35
117	0 66	0 4	0 42	64
118	0 62	0 25	0 41	66
1 2	0 41	0 15	0 26	63
1 3	0 25	0 14	0 11	44
124	0 23	0 10	0 13	57
Mean				55
		Insulin added in all flasks		
119	6 38	2 64	3 74	59
120	6 34	2 91	3 43	54
121	2 91	1 1	1 79	62
125	9 59	5 00	4 59	48
126	4 19	2 39	1 80	43
127	8 48	4 8	3 66	43
Mean				52

* See footnot Table I

cortical effect upon *hepatic* lipogenesis may well be secondary to other hepatic metabolic events induced by glucocorticoid administration or withdrawal

I should like to devote the last minutes of this presentation to two as yet somewhat unrelated observations which I would use mostly to emphasize the probably multiple nature of the regulatory effects exerted upon adipose tissue effects which we are only just beginning to analyse I am fortunate in being able to present work from both present and past collaborators some very recently obtained First

Albert Winegrad now working in the laboratories of Dr Stadie Dr Lukens has recently expanded observations begun jointly on the effects of bovine growth hormone on adipose tissue *in* ? We had previously found that growth hormone added *in* ? stimulates glucose uptake stimulates CO_2 production but



IC 5 Adipose ti ue a d liver li es were obt ed from th *sam* animals Note that only ti values b t n t bsol te valu s should be compared between tis es sin the conons of th incubat ns with regard t tim as well as the reference mea urem t diffe ed br ket djoini g ea h col mn indicates th t nd d of error of the mean for each p (unp bli hed d ta of Jeanrena d a d Renold)

ls to stimulate lipogenesis When as shown in Fig 6 the oxidan of carbon-1 and carbon-6 was measured in the same tissue at urly intervals for 4 hours unstimulated tissue persistently proced more CO_2 from carbon-1 than from carbon-6 and the dition of insulin at the end of the first hour resulted in a much eater increase of CO production from carbon-1 than from rbon-6 as expected from observations already discussed (Table I) hen growth hormone however was added after the first hour apparently selective stimulation of the oxidation of carbon-6 sulted A possible explanation of this rather unexpected finding

would suggest a specific stimulation of the glucuronic acid pathway * It is necessary to point out the high concentration of growth hormone required to exert this effect upon this tissue

Secondly Drs Peter Bally and George Cahill in our laboratory have studied the effects of glucose upon the incorporation of palmitate-1-^{14}C into adipose tissue triglyceride and upon its oxida

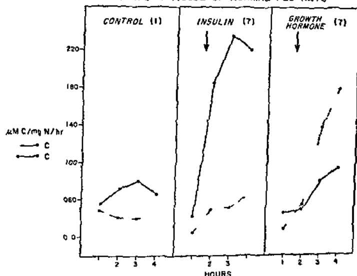


Fig 6 Each point represents the hourly rate of $^{14}CO_2$ production In the case of insulin and growth hormone the addition of either hormone immediately followed the CO_2 collection at the end of the first hour Incubation was carried out in Krebs phosphate buffer (observations of Winegrad Shaw Lukens and Stadie)

tion to CO As illustrated in Fig 7 the presence of glucose at quite low concentrations increased fatty acid storage but decreased fatty acid oxidation ** Also Gordon recently reported a direct insulin effect upon fatty acid *release* by adipose tissue *in the presence of* glucose

* These studies have since been published by *Winegrad A J Shaw W Stadie W C Luken F D and Renold A E (1959) J biol Chem* 234 192

** These studies are now in process of publication by Bally P R Cahill G F Jr Leboeuf B and Renold A E (1960) *J biol Chem*

I should like to begin the summary by pointing out that according to the schema prevailing 10 or 15 years ago glucose (with regard to its relations to fat metabolism) was primarily transported as glucose to the liver there transformed into fatty acids transported as fatty acids or triglycerides to adipose tissue for storage recalled

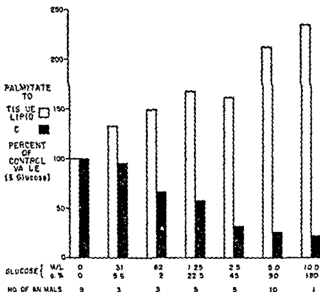


Fig 7 In ubation carried out in Krebs b carbonate buffer with 5 per cent salt free human albumin and 2 mEq /l palmitate-1-14C Note that the values are expressed as per cent of the control value obtained in the absence of glucose (data of Bally Cahill LeBœuf and Renold)

when needed to the liver and there transformed into ketone b and thus finally made available for general consumption by such as muscle In accord with newer knowledge this schema b en largely replaced by direct fatty acid synthesis from glucose *adipose tissue itself* and direct utilization of fatty acids released adipose ti sue not only by the liver but also (and to a major by tissues such as muscle in particular heart muscle This being

should we not really expect a highly sensitive regulatory system for fatty acid synthesis fatty fatty acid release by this tissue? We believe that to be the case and feel that we have found as evidence of a direct action of at least one hormone adipose tissue an action exerted upon glucose synthesis glucose oxidation and fatty acid magnitude of the effect of insulin upon lipogenesis ' and the low concentration of insulin required may well be the *main* site of insulin action upon ' data we have gathered to date are for the time b with a primary insulin effect upon this tissue at an glucose utilization specifically between extracell intracellular glucose-6-phosphate while not of co other sites of action on parameters not measured As made no distinction between an effect of insulin transport or phosphorylation

REFERENCES

1 BELOFF-CHAIN A CATANZARO R CHAIN E R MASI, I & P *Selected Sci ntific P p s f om the I t t ito Superio e di San t* 1 Part 3 345
2 BLOCH K & KRAMER, W (1948) *Cold Spring Harb r Sympos a, Quant*
3 BRADY R. O & GURIN S (1950) *J biol Chem* 187 589
4 BRADY R O LUKENS F D W & GURIN S (1951) *J biol Chem*
5 FAVARGER P & GERLACH J (1955) *Hel Pl ys ol et Pl arma I Acta* 13 96
6 FAVARGER P & BODUR H (1956) *J Phys ol (Paris)* 48 534
7 HAUGAARD N & MARSH J B (1952) *J b ol Chem* 194 33
8 HAUSBERGER F X MILSTEIN S W & RUTMAN R J (1954) *J bi l Chem*
9 JEANRENAUD B & RENOLD A E (unpublished observations)
10 KRAHL, M E (1951) *Ann N Y Ac d S i* 54 649
11 LANGDON R G (1957) *J b l Chem* 226 615
12 MARTIN D B RENOLD A E & DAGENAIS Y M (1958) *Lancet* ii 76
13 MILSTEIN S W & HAUSBERGER F X (1956) *D b tes* 5 89
14 MILSTEIN S W (1956) *Proc Soc Exptl B l M d* 92,
15 RENOLD A E MARBLE A & FAWCETT D W (195 55
16 RENOLD A E WINEGRAD A. I & MARTIN D B (*Acta* 24
17 SHAPIRO B & WERTHEIMER E (1956) *Met b lism*
18 SHAPIRO B (1957) *In Prog ess i t e Chemistry f Fats* 196 (
Press New York)
19 SIPERSTEIN M D & FAGAN V M (1957) *Sc ence*
20 WERTHEIMER E & SHAPIRO B (1948) *Phys ol*

2

INSULIN IN SERUM PROTEIN FRACTIONS

K W Taylor*

Department of Biochemistry University of Cambridge

Measurements of the glucose uptake of the isolated rat diaphragm have now been shown by manv workers to be the basis of a sensitive mode of insulin assay *in vitro* However when the method is applied to blood its reliability may be seriously affected by the presence of other substances in blood This is especially the case where the blood serum of diabetic man or animals is concerned Thus Randle and Young [15] and Bornstein [7] have described lipo-protein type inhibitors of glucose uptake by the diaphragm in diabetic men or animals Field and Stetten[9] claim to have detected an α globulin inhibitor of insulin action in the serum of ketosed human diabetics and more recently Vallance-Owen *et al* [18] have described an insulin inhibitor present in the serum albumin of patients with uncontrolled diabetes According to Groen[10] and his co-workers adrenaline too may inhibit the action of insulin even when present in concentrations which are stated to be physiological

There are moreover many substances which may by themselves simulate the action of insulin thus certain small molecule substances such as salicylate or dinitrophenol are now known to increase glucose uptake by the diaphragm and serum albumin has been reported to do the same [13 14]

A further difficulty relates to the form in which circulating insulin is present in blood From the studies of Berson *et al* [4] it seems likely that some of the insulin present in the serum of insulin-treated diabetics may be bound to globulin by what is apparently antibody and this antibody bound insulin may not be necessarily available for action on the diaphragm It is also possible that not all the insulin in normal serum is biologically available when undiluted serum is employed and tested on the diaphragm

These considerations led to an attempt to fractionate serum protein for insulin activity The method which we have found most

* Elmor Research St dent

valuable has been zone electrophoresis on treated cellulose columns In general 5 ml of serum have been electrophoresed for 40 hours at 1000 volts in a phosphate-borate buffer pH 8 4 After electrophoresis the separated proteins were eluted from the column and the protein concentration of 5 ml portions of the eluate determined by measuring the optical D at 280 m/μ in the Beckman spectro photometer In order to locate insulin in the serum protein fraction a trace labelled ^{131}I insulin has been prepared and electrophoresed in the presence of serum The results show that a peak of radio activity appears migrating somewhat slower than the albumin fraction This confirms the observations of several other groups of workers that free insulin migrates on starch blocks or paper with a mobility slightly less than that of serum albumin at or about this pH

In these experiments especial attention was paid to the possibility that the insulin itself might have been damaged by iodination so that its electrophoretic mobility was altered since Berson[5] has reported that this type of damage may take place using radio-iodine The preparation obtained however had a similar mobility on electrophoresis to that of un-iodinated insulin and behaved similarly when subjected to paper chromatography in a butanol/acetic system

The next series of experiments were concerned with the effect on the glucose uptake of the diaphragm of serum fractions obtained by electrophoresing serum mixed with excess unlabelled insulin In this experiment fractions were dialysed against distilled water freeze-dried and the dry protein residue dissolved in buffered glucose before testing on the diaphragm A maximal effect is obtained with the slower moving albumin fractions with some smaller effects on the β and γ globulins (see Fig 1)

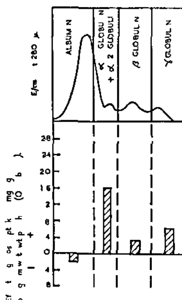
When normal serum was electrophoresed without additions the greatest effect was found again in the slower-moving albumin fractions with somewhat lesser activity appearing in the β and γ globulins These results have been reported in full elsewhere[16 17]

Broadly similar results have been obtained by testing fractions made by electrophoresing the serum of diabetic patients treated with insulin stimulation being obtained in the slower- but not th faster-moving albumin fractions and also in the β and γ globulins

In recent publications both Beigelman[3] and Bolinger *et al*[6] have independently reported that insulin activity is present in the β- and

γ-globulin fractions obtained by the electrophoresis of normal human serum though some doubt seems to exist as to whether or *not albumin may also exhibit this on occasions*

In a general sense these experiments agree with those reported above although the effects we have noted in the albumin zone appear to be somewhat greater than theirs



F G 1 The ff t of um pro in f ti m de by zon l t ophore s on the gl o ptake f the sol ted r d ph gm *tro* Ex in ulin added to m b f e e tro- ph e

Since labelled and unlabelled insulin added to serum migrate in the slower moving albumin zone it seems probable that the activity found in this region when serum to which additions have been made is electrophoresed is due in fact to free insulin present in the serum However some doubt as to the exact nature of the stimulating effect due to the β and γ globulin remained and it was decided therefore to see if this effect was neutralizable by insulin antiserum preparations Since human insulin was not available it was decided to prepare antiserum against ox insulin and to test this against ox-

serum protein fractions Antisera to ox insulin were prepared in guinea-pigs by the method of Moloney and Coval[L] using an emulsion of insulin in oil Such preparations will completely neutralize the effect of insulin in stimulating the glucose uptake of the isolated rat diaphragm Control preparations made by injecting oil alone into guinea-pigs did not neutralize insulin Neutralization of the effects of the insulin on the rat diaphragm have also recently been reported by Birkenshaw *et al*[8]

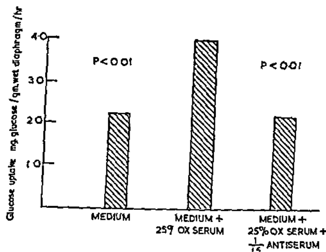


FIG 2 The effect of antiserum to ox insulin prepared in guinea p gs on the stimulation by ox serum of the glucose uptake of the isolated rat diaphragm

It was first decided to attempt to neutralize the effects of whole ox serum by the antiserum preparation In Fig 2 are shown the results of such an experiment

The ox serum was tested in 25 per cent dilution with an antiserum concentration of 1 in 15 Complete neutralization of the stimulating effect of the serum has been obtained These results have been obtained with other specimens of ox serum

The results of electrophoresing ox serum in parallel with the effects of various fractions on the glucose uptake of the isolated rat diaphragm are shown in Fig 3

For purposes of investigating effects on glucose uptake broad divisions of fractions have been made into albumin and post-albumin zone and β and γ globulins It will be seen that while the whole

albumin fraction has no effect on glucose uptake there is a small effect in the post-albumin zone a greater effect in the β globulins and the greatest effect in the γ globulins These effects appear to be neutralizable by anti-sera as can be seen in Fig 4 using another sample of antiserum

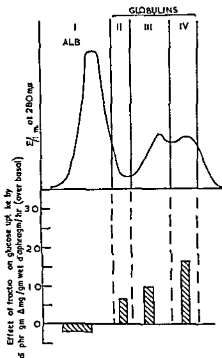


FIG 3 The eff t of fr n ns m de by the z n lectroph sis of no mal ox serum on th glucose uptak of th olated t diaphr gm.

In the left half of the figure are shown the neutralizing effects of antiserum on the β globulins and post albumin fractions and on the right are shown the effects on the γ globulins From these results therefore it seems that all the effects of ox-serum fractions on glucose uptake are neutralizable by ox insulin antisera Similar results have been obtained with the γ globulins prepared by electrophoresis from several other ox-serum samples

Complete neutralization of the effects of the whole serum of an insulin-treated diabetic patient have also been obtained as well as neutralization of the γ globulin of the same patient It seems therefore that if the antiserum test is a specific one these γ globulin effects are due in fact to insulin

I would like now to return to the effects of serum albumin itself on glucose uptake Several workers have in the past reported that serum albumin enhances the uptake of glucose by the isolated rat

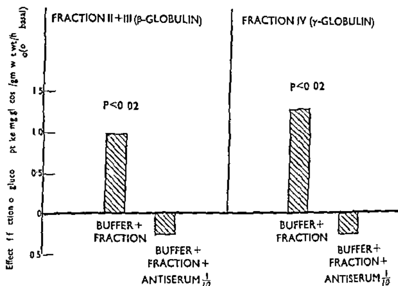


FIG 4 The neutralization of the insulin effect of ox serum protein fractions by antiserum to ox insulin prepared in guinea pigs

diaphragm and it has been suggested by Randle[14] that this effect might be due to a contamination of the albumin by insulin Column electrophoretic experiments support this conclusion In these experiments the whole albumin fraction of ox serum and ... faster-moving fractions of human albumin do not exh... —
indeed there is if anything a slight inhibition
Albumin from several species however made I
precipitation method of Korner and Debro[11]
uptake of the diaphragm — the effect being
greatest in ...

In Fig 5 are shown the effects of human albumin prepared by this method on glucose uptake compared with the effects of albumin derived from human serum to which a small quantity of insulin had been added before precipitation In this case the albumin preparations were precipitated with trichloracetic acid extracted with alcohol dialysed against distilled water lyophilized and after making up in buffered glucose were tested on the diaphragm A small effect is seen with the sample made from serum without

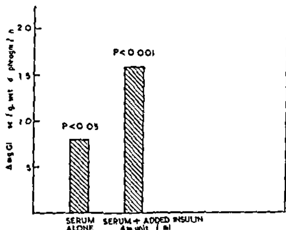


Fig 5 The effect of albumin prepa ed by th fr ctional p ecip t t n of proteins f o n normal serum on the gl cose uptak of the isolated at di phr gm

additions and a very much larger effect with the sample to which insulin had been added These results indicate that insulin may be co-precipitated with serum albumin under these conditions Albumin itself appears to be without insulin activity and indeed the results of the work of Vallance-Owen *et al*[18] indicates that an antagonist may be present in this fraction

Before drawing some general conclusions mention should be made of more recent work on the serum of insulin treated diabetics It was noted in some experiments that the effect of the γ globulin fractions obtained by electrophoresis of serum of these individuals was considerably greater than normal even though the whole serum itself had no statistically significant effect on glucose uptake

These results led to an attempt to extract insulin from this type of serum by an acid-alcohol method After dialysis against water freeze-dried extracts were made up in buffered glucose and tested on the diaphragm The results of such an experiment showing a large γ-globulin effect are shown in Fig 6 In this case while the unfractioned serum is without effect on glucose uptake a larger effect is obtained with the extract These results confirm the work of Baird and Bornstein who have shown by a similar extraction technique that insulin may be present in the serum of such individuals though its biological activity may in some way be masked

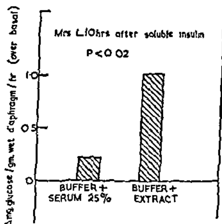


Fig 6 The effect of serum and crude acid-ethanol extracts of serum of an insulin treated diabetic patient on the glucose uptake of the isolated rat diaphragm

To summarize it seems that in whole serum all the effects in stimulating glucose uptake are due to insulin Likewise the stimulating activity of plasma protein fractions seems also to be due to insulin if insulin antiserum represents a specific insulin antagonist We have found no evidence that albumin itself free from insulin stimulates the uptake of glucose by the diaphragm

Electrophoresis of human serum has revealed stimulatory effects on glucose uptake in two zones namely the slower-moving albumin zone and the β and γ globulins Since free insulin labelled or unlabelled added to blood appears to migrate in the slower-moving albumin zone the effect observed in this zone in normal serum after

electrophoresis would seem to represent free circulating plasma insulin

Studies with antisera al o suggest that the effects noted in the β and γ globulins are due to insulin If this is so then this insulin may be associated in some way with the globulins Berson[4] has suggested that globulin-bound insulin is present in the serum of insulin-treated diabetics and it is not impossible that similar association of insulin with β and γ globulins takes place even in normal serum The idea that insulin may in some way be associated with serum proteins is not entirely new Using ion exchange columns Antoniades *et al*[1] has also reported that insulin added to blood may behave differently from the circulating hormone and it has been suggested that this may be due to the association of insulin with serum proteins

Finally the possibility that some of the blood insulin of treated diabetics may be bound to γ globulins and therefore biologically unavailable may be a clinically important one and this dese full investigation

ACKNOWLEDGMENTS

Professor F G Young has my best thanks for help and encoι ment throughout the investigations

I would also like to record my thanks to Dr I B Cole A brookes Hospital Cambridge for permission to use data on of his patients and to the British Diabetic Association and of the Elmore Research Fund for grants towards my expenses

REFERENCES

1 ANTONIADES H N BEIGELMAN P M PENNELL R B THORN G W & L (19 8) *Metabo sm* vii 266
2 BAIRD C W & BORNSTEIN J (1957) *Lancet* i 1111
3 BEIGELMAN P M (1958) *Proceed ngs of 3 d Internat on l Diabetic Fed Co g ess*
4 BERSON S A & YALOW R S (19 7) *D abet s* 6 402
5 BERSON S A YALOW R S BAUMAN A ROTHSCHILD M A & N *J Cl I* e c 35 170
6 BOLIN ER, R E VAN DER GELD H WILLEBRANDS A F & GROEN J *T ... hr Geneesk* 102 700
7 BORNSTEIN J (1953) *J biol Chem* 205 513
8 BRAKENSHAW V J GURD M R RANDALL S S CURRY A S PRICE, D P H (1958) *Brit med J* ii 463
9 FIELD J B & STETTEN D Jnr (1956) *Amer J M d* 21 33
10 GROEN J VAN DER GELD H BOLINGER R E & WILLEBRANDS A F 7 273
11 KORNER, A & DEBRO J R (1956) *Natu e* 178 1067
12 MOLONEY P J & COVAL, M (1955) *B ochem J* 59 179

13 PARK C R 1952) *The Phosphorus Metabolism* Vol II p 634 Eds W D McElroy and B Glass Johns Hopkins Press Baltimore
14 RANDLE P J (1957) *C ba Foundat on Coll E docrinol* 11 115
15 RANDLE, P J & YOUNG F G (1957) *The Hormonal Regulat on of Energy Metab l sm* p 91 Ed L W Kinsell publ Charles C Thomas Springf eld Illinois
16 RANDLE P J & TAYLOR K W *J Endocrinology* (In press)
17 TAYLOR K W (1958) *B ochem J* 69 59
18 VALLANCE OWEN J B DENNES E & CAMPBELL P N (1958) Lancet ii 336

BIO-ASSAY OF SERUM INSULIN-LIKE FACTORS*

PAUL M BEIGELMAN

University of Southern California School of Medicine

There has been abundant evidence that alterations in adipose-tissue metabolism are induced by insulin [4 5 8] More recently it has been noted that increments of glucose uptake by adipose tissue are responsive to such small concentrations of insulin that an insulin bio assay sensitive to 10 micro-units/ml may be feasible with this system [2 6] For the past year this technique of bio-assay for insulin-like activity utilizing glucose uptake by wistar rat epididymal adipose tissue has been studied at the University of Southern California Medical School This method has proved highly sensitive and quite simple but does not appear as yet to be very precise

METHODS

Fed male Wistar rats were stunned by a blow to the head and quickly killed by decapitation Segments of epididymal adipose tissue were rapidly excised quickly weighed in a torsion balance and immediately added to Krebs bicarbonate buffer containing glucose Human sera and concentrations of insulin were added to the buffer and glucose substrate 0 [illegible] per cent albumin was incorporated with the controls and the insulin determinations Incubation proceeded for 2-4 hours in a Dubnoff metabolic shaker under 95 per cent O -5 per cent CO_2 at 36 5 C The usual total glucose was 0-3 0 mg in 0-[illegible] 5 ml volume of buffer per beaker Residual glucose was measured by the Somogyi-Nelson method [7] and glucose uptake expressed as mg glucose/g adipose tissue

RESULTS

A number of variables including weight of the animal initial glucose concentration duration of incubation weight of adipose tissue state of nutrition and protein concentration are being studied At present the weight of the animal appears to be the most impor-

* This work was s ted in pa t by G t No A 1516 of the U S P H.S d a g nt by the D b tes Ass ci ti of South California

tant factor Higher glucose uptakes and an improved response to the insulin were associated with employment of adipose tissue from lower weight animals The correlation coefficient (r) for animals in the 100–180 g weight range was 0 54 ($P < 0\ 001$) Conversely the r value was 0 22 ($P > 0\ 4$) for rats weighing more than 350 g (Fig 1) Suggestive loss of insulin sensitivity also appears to be associated with increased weight of tissue and decreased duration of incubation Preliminary evidence indicates that alterations of initial

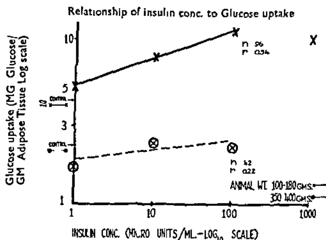


Fig 1 Relation hip of glucose uptake mg glucose/g adipose tissue expressed as $\log_{10}$ to insulin concentrations log values (all values on graph represented as anti l gs) of 1 10 100 and 1000 m cro-units/ml Control uptake also represented Rel tionship of increment of glucose uptake to insulin concentration evident in 1 100 micro-units/ml range Correlation *coefficient (r) calculated for this dose range*

glucose concentration within the range 2 0–4 0 mg do not materially affect bio-assay sensitivity There is some suggestion of slight insulin-like activity associated with albumin concentrations of 1 0 per cent or greater but the evidence is not conclusive At present 40–60 mg segments of adipose tissue from rats weighing 100–180 g are incubated for 2–3 hours in Krebs bicarbonate buffer containing 2–3 mg of glucose per beaker

Clinical studies have been performed on normal and diabetic coma sera There is considerable individual variation of serum (diluted 1/4 or 1/10) insulin-like activity in fifteen normal subjects studied To some extent this may reflect ' variability

of the method but also indicates wide fluctuations of normal serum insulin-like activity Preliminary evidence suggests that 2-hour post-prandial serum insulin-like activity is greater than that of corresponding fasting sera

Serial serum insulin-like activity determinations have been performed on eight diabetic coma patients[3] The initial pre-treatment coma specimens demonstrated usually little or no insulin-like activity Rapid increment of serum insulin-like activity accompanied exogenous insulin therapy and did not appear to be associated with any alteration of the patients clinical status Certainly there was no very apparent relationship between the increase of serum insulin like activity and change in blood glucose levels or CO_2 combining power

Investigation of insulin-like activity in various discrete serum protein fractions prepared by the method of continuous flow paper electrophoresis was performed in this bio-assay system The technique of continuous flow paper electrophoresis has been described[1] Serum albumin γ globulin and α_2 globulin definitely demonstrated no insulin-like activity β globulin and adjacent fractions particularly the fraction migrating between β and γ globulins were associated with increased glucose uptakes The fraction which migrated between β and γ globulins possessed definite and significant insulin like activity

Conclusions

The method of bio-assay for insulin-like substances utilizing glucose uptake by rat epididymal adipose tissue is sensitive to 10 micro-units of insulin/ml and sensitivity on occasion has been noted to as little as 1 micro-unit of insulin/ml This technique is relatively simple but the method does not possess a very high degree of precision which may be an inherent characteristic of this being a protein hormone bio-assay Considerable refinement of this method is possible as various modifying factors are corrected The animal weight appears to be a factor of primary importance the bio-assay sensitivity being inversely proportional to the weight of the rat

Clinical studies indicate considerable variation of insulin-like activity in normal sera Pre treatment diabetic coma sera possess little or no insulin like activity Marked increase of activity is

associated with exogenous insulin administration but does not appear to correspond to clinical response

Studies by this method of serum protein fractions obtained by continuous flow paper electrophoresis indicate predominant insulin like activity in the β-globulin area These observations are in accord with the results obtained by testing similar fractions in a crude insulin-assay system utilizing blood glucose decrement of intact anaesthetized mice[1]

It is of the utmost importance to recall that this method as with other techniques of bio-assay does not specifically measure insulin but only expresses a non-specific change namely glucose dis appearance It is probably justified to make the assumption that this reflects uptake of glucose by the adipose tissue and one may even be permitted to equate glucose uptake with insulin-like activity However to attribute such an effect solely to insulin would be an error and the term insulin-assay therefore is a misnomer Multiple insulin-like factors some possibly unrelated to insulin may be operative in a fashion similar perhaps to the several anti-insulin or insulin-inhibitory factors which have been characterized[2]

Summary

A method of bio-assay for insulin-like activity utilizing glucose uptake by rat epididymal adipose tissue has been developed which is sensitive to as little as 10 micro-units of insulin/ml This technique has been applied to clinical studies and suggests wide variability of normal serum insulin-like activity Pre-treatment diabetic sera demonstrate little or no insulin-like activity Serum protein fractionation *studies indicate that insulin-like activity is* associated with β globulin or adjacent globulins

REFERENCES

1 Beigelman P M (1958) *Diabetes* 7 365
2 Beigelman P M & Antoniades H N (1958) *Metabolism* 7 269
3 Beigelman P M (1959) *Diabetes* 8 23
4 Hausberger F X & Milstein S W (1955) *J biol Chem* 214 483
5 Krahl M E (1951) *Ann N Y Acad Sci* 54 649
6 Martin D B Renold A I & Dagenais Y M (1958) *Lancet* ii 75–6
7 Nelson N (1944) *J biol Chem* 153 375
8 Renold A E Marble, A & Fawcett D W (1950) *Endocrinology* 46 574

DISCUSSION

WRIGHT I find it rather hard after these three papers ground which I can use as a basis for opening this heard about the use of two *in vitro* systems and they in different ways and with different ends in view I w to be thoroughly selfish and talk about what interests applicable I think to the remarks made by the last two

They have referred to biologically active substances wh in effect in the epididymal pad and on the rat Dr Taylor has made a positive attempt to identify the n material When we are considering substances or ec extracts which might contain insulin we must consider t might be used to establish the identity of that substance

It is unlikely that purely chemical or physical methods to the identification of small amounts of insulin mixed or c relatively large quantities of contaminating proteins in body as plasma or their extracts The biological properties of insu be used for its detection and assay and it has to be shown tl logical responses produced by suspected material *in vitro* and identical with those of insulin

Many substances other than insulin are known to exert hyp effects in animals amongst these are the sulphonyl ureas phenethyl biguanide Others which may or may not exert caemic effects in animals will stimulate glucose consumption isolated rat diaphragm synthalin phenethyl biguanide arsenite etc fall into this category It may be possible to other biologically active substances by observing the pattern of logical response produced thus phenethyl biguanide causes a crease in lactic acid production in the rat diaphragm which is r in the case of insulin By such methods one may be able to insulin as the cause of biological activity in an extract but more po evidence is necessary to identify insulin as the active co activity is destroyed after treatment with cysteine pepsin, insul alkali if it is concentrated by extraction of the tissue by a method des to extract insulin or if it moves under electrophoresis at a rate sim that of insulin it is likely that the active component is in fact in Now another aid can be used which is more specific in its action involves a simpler technique

Moloney and Coval (*Biochem J* 1955 59 179) have shown that insulin is injected repeatedly into guinea pigs serum from such an when mixed before injection with insulin will protect mice from convulsive action of the hormone I have found that if serum (- 5 cent v/v) from guinea pigs sensitized in the same way to purified bovir

insulin is added to the incubation medium at the same time as insulin (approx 1 milli-unit/ml) it will abolish the stimulant effect of the hormone upon glucose consumption by the isolated rat diaphragm normal guinea-pig serum has no such action Serum from animals sensitized to bovine insulin will also abolish the stimulant actions of similar concentrations of pig sheep and human insulin (Fig 1) It has no effect in similar concentration upon the stimulant actions of synthalin (5 mg /100 ml) or phenethyl biguanide (50 mg /100 ml) Moloney and Coval concluded that such serum contains antibodies and it appears that they are specific though not species specific for insulin I have no evidence which would contradict this

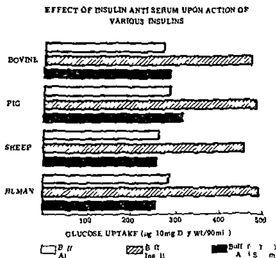


FIG 1

Serum from guinea-pigs sensitized to bovine insulin has been used to aid in the identification of insulin in plasma and its extracts It is known that plasma and some of its protein fractions exert a hypoglycaemic effect in ADH and ADHA rats and as shown by Dr Beigelman and Dr Taylor that they stimulate the glucose consumption of the isolated rat diaphragm and of the epididymal fat pad In the case of bovine plasma and one of its protein fractions (Armour Fraction V) I have made similar observations using the isolated rat diaphragm and have shown that in both cases the stimulant effect is abolished by treatment with cysteine or with the insulin antiserum (Wright *Proceedings of 3rd Congress*

of International Diabetic Federation (in press)) normal
has no effect

Recently a man was convicted for the murder of his
insulin (Birkinshaw *et al* *Brit med J* 2 463) The
underlying injection marks found on the dead woman
extracted by the normal method used commercially for
from the pancreas These extracts were injected into
to have a hypoglycaemic effect equivalent to that of 80
This hypoglycaemic activity was abolished after treatmen
with insulinase or cysteine and the biological response th
guinea pigs was the same as that of an equivalent dose of
the rat diaphragm I found that these extracts stimulated
sumption and that their activity was reduced or abolished
with cysteine or pepsin I was also able to show that the
serum but not normal serum from guinea pigs abolished
like activity of the extracts

These experiments lead me to believe that if the biolc
a tissue or its extract can be abolished by treatment with
insulin sensitized animals then the active material present
However the converse may not necessarily be true where
activity is not destroyed it is still possible that insulin may be the
agent

RUSSELL FRASER The papers this morning have clearly
important next step to explore the uses of these bio-assay pro
have shown already how far this procedure has gone Dr P
is of course throwing a new light on the relevance of obesity to
I do not know whether he would like later to comment on any
he has got that insulin may influence the release of glucose fr
adipose tissues in disease or in normal circumstances

In regard to the insulin in serum fractions it still seems a little
to be sure but perhaps some of the sites as with many other h
are more specific than others

I have much pleasure in throwing these and other points op
discussion

CHAIN I should like to comment on Dr Renold s paper In our v
the strong effect of insulin on fat synthesis supports the view that ins
steps up the energetic potential of the cell In the case of a
energy requiring action such as the fatty acid synthesis this must
accompanied by a concomitant increase in oxygen uptake oth
there would be no possibility of deriving the energy for this fatty
synthesis

The effect of insulin on fatty acid synthesis has of course been
served in various tissues including the lactating mammary glands

fact Dr Folley and his group have used the strong effect on the increased respiratory quotient in that system as an *in vitro* measure of insulin activity It seems to me a very sensitive method for assaying insulin and it has not been mentioned at all this morning

An increased oxygen uptake cannot be obtained by increasing glucose concentration and here again we have got an insulin effect which cannot be simulated by glucose concentration I should say the situation resembles that which Briton Chance has seen in his system where he can get an increased oxygen stimulation by supplying free ADP As long as

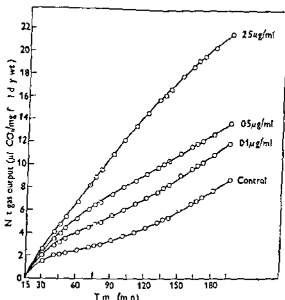


FIG 2 Manometric *in vitro* assay of insulin band on the measurement of the extra CO output produced by insulin in lact ting mammary gland tissue (From *Selected Scientific Papers from the Istituto Superiore di Sanita* 1956 1 Part III p 440 Present knowledge of the mechanism of the mode of action of insulin by Chain *et al*)

ADP is phosphorylated up goes the oxygen which means in other words as long as there is an energy demand on the part of the cell it is supplied by the response of oxygen As soon as the ADP is fully transformed into ATP the oxygen consumption goes down I think it is when one sees an increase of oxygen by adding a substrate to the tissue provided of course saturation level is reached it means one of two things either the cell is carrying out mechanical or chemical work or it is cheated into believing that there is a demand for energy requirement by an uncoupling mechanism You see this with dinitrophenol and so on. The tissues in

which fat synthesis takes place such as this white adipose tissue or the lactating mammary gland tissue provide we believe an ideal system for studying the connection between the respiratory chain and the coupling to the synthetic reactions in the particular case of the brown adipose tissue two synthetic reactions fat synthesis and glycogen synthesis are accelerated by insulin I was interested to hear that this problem is being studied by Dr Ball we are engaged in similar studies trying to use spectrophotometric methods

This is the monometric method based on the increase of the respiratory quotient This system responds to 0 1 γ per ml of insulin I thought it would be interesting for you to see it

TABLE I

LOG DOSE RESPONSE CURVE FOR THE EFFECT OF INSULIN ON THE INCORPORATION OF C GLYCINE INTO DIAPHRAGM PROTEIN IN VITRO

I In nc t t on (m ll-u ts/ml)	*Effect f In M d fference ± S E of d ff ence (Δ co nts p m n p t)*	*S g fica f d ffere (P)*
0 0125	−1 ± 9 3	
0 05	37 ± 13 4	<0 2
0 20	71 ± 13 4	<0 001
0 80	99 ± 10 8	<0 001
3 20	113 ± 12 5	<0 001
12 80	1 6 ± 13 4	<0 001

Ea h val e is the mean of twelv d termin ti ns
Incorporation (counts per min) in th b ence f insulin v 258 ± 4·5

MANCHESTER I would like to mention another insulin bio-assay that Dr P J Randle and I have developed I pointed out in my paper yesterday that insulin at concentrations as low as 0 5 milli unit/ml (2 5 mμg /ml) stimulates incorporation of amino acids into protein of isolated rat diaphragm In Table I is shown a log dose response curve (the combined results of two experiments performed by Dr Randle and myself) for the effect of insulin *in vitro* on the incorporation of ^{14}C glycine into the protein of isolated rat diaphragm The concentration of insulin was varied in fourfold steps over the range 0 0125 12 80 milli units/ml increase in the concentration of insulin added consistently increased the magnitude of the stimulation of incorporation produced by insulin over the range of insulin concentrations studied

Analysis of regression of results of the two experiments contained in Table I is shown in Table II The two experiments are here treated separately in both cases regression is highly significant and the deviation from regression very small The regression is therefore almost certainly linear Since the slope of the log dose response curve is shallow (the

TABLE II

ANALYSIS OF REGRESSION FOR THE EFFECT OF INSULIN ON THE INCORPORATION OF ^{14}C GLYCINE INTO DIAPHRAGM PROTEIN IN VITRO

Exp no	*Source of variation*	*Mean square*	*F*	*P*	*Slope ± S E of slope*	λ
1	Linear regression	39 647	22 3	<0 001	4 7 ± 6 7	0 39
	Deviations from regression	764	0 43	>0 05		
	Random sampling	1775				
2	Linear regression	20 093	13 9	<0 01	30 4 ± 6 3	0 51
	Deviations from regression	533	0 37	>0 05		
	Random sampling	1446				

maximum increase of incorporation that can be produced by addition of insulin is only of the order of 50 per cent of the basal level — Manchester and Young *Biochem J* 1958 70 353) the degree of precision of the assay (λ) is not greatly superior to other existing insulin assays

The insulin activity of two samples of human plasma collected from normal people 2 hours after oral glucose has been measured by this method The results 2 milli units/ml and 10 milli units/ml (Table III) are of the same order as have previously been obtained by Dr Randle (Randle *Ciba Colloquia Endocrinology* 1957 11 115) by the use of the glucose uptake of isolated rat diaphragm as his assay

TABLE III

MEASUREMENT OF PLASMA INSULIN ACTIVITY BY ITS EFFECT ON THE INCORPORATION OF ^{14}C GLYCINE INTO DIAPHRAGM PROTEIN IN VITRO

Counts per minute in protein Mean ± S E of mean					
	Control	*Insulin (milli units/ml)* 0 24	1 0	*Plasma fourfold dilution*	*Calculated insulin activity of plasma (milli-units/ml)*
Assay 1	249 ± 8	300 ± 10	315 ± 7	323 ± 5	10
Assay 2	327 ± 17	443 ± 16	489 ± 28	468 ± 30	2

Each figure is the mean of eight observations
Blood samples collected from normal people 2 hours after oral glucose (50 g)

STEWART I was recently reading some early literature and found that Best and his colleagues many years ago had extracted the blood insulin or an insulin-like substance with the mean activity of 9 3 milli units/c c Even in the diabetic animal there was a level of 16 0 milli units on average

We are talking nowadays in terms of *micro-units* of insulin and I want

to ask Dr Taylor whether he had actually assayed t
he had prepared If these substances are not insulin th
potent materials

TAYLOR In answer to Mr Stewart I would say that
any very exact quantitative work yet on acid alcohol
there is no doubt that with the kind of procedure we r
down to a level say of 1 milli unit/ml in an extract
up and will certainly come through the extraction proced
I cannot say more about the quantitative nature of this
I do not know why Best got these very high figures I
explanation for that

RENOLD I wish to thank Professor Chain for his ec
mammary gland work which he referred to is of
although it should be pointed out that the lowest effective
of insulin in this tissue is 1,000 µg/ml as compared to the mini
concentration of 10 µg/ml for adipose tissue Balmain herse
from these studies that the system was inadequately sensitive
for the bio-assay of insulin in serum With regard to the
significance of the observation in mammary gland it may be
calling that the effectiveness of insulin is limited to *lactating* rat
gland and was not apparent in sheep mammary gland
I have no comments concerning the nature of the energizir
effect postulated by Professor Chain The effect of insulin
consumption in this tissue is surprisingly small and indeed
significant even when major acceleration of glucose utiliz
demonstrated

In closing I may perhaps be permitted to say that I was a li
appointed by the limitation of the discussion to the insulin bio
problem For me the application of our observations in ad
to the bio-assay of insulin like activity is really rather incidental
fascinated however by the physiologic implications of this
so highly sensitive responsiveness of adipose tissue to insulin and I
have been anxious to hear the comments of this group on this
general topic

BEIGELMAN Evidence has been presented at this symposium
that plasma insulin like activity is definitely due at least in part to
insulin However other factors may be present in plasma which
have insulin like activity Walaas *et al* in Oslo Norway have obtain
by dialysis a substance from normal plasma which appears to be
amino acid or peptide is definitely not insulin and has insulin li
activity Following extraction of this material there was no resid
insulin like activity in the plasma. Factors such as this may be of
physiological or pathological importance

PART VII

INSULIN ANTAGONISTS

Chairman PROFESSOR T RUSSELL FRASER

INSULIN ANTAGONISTS IN DIABETIC PLASMA

J VALLANCE-OWEN

University of Durham Medical School
King s College Newcastle-upon-Tyne

The rat diaphragm technique that we have published and used for estimating plasma insulin activity [6] and through it any insulin antagonism in the plasma of diabetic patients is basically similar though differing somewhat in practical detail from that used by Groen[4] and Randle [5]

Probably the most important difference is in the handling of the plasma itself We compare the glucose uptake from undiluted plasma with that achieved by the buffer solution with and without added insulin We ordinarily find significant differences between the lowest concentration of insulin tested namely 10 micro units/ml and the basal level which is the amount of glucose taken up by the diaphragm when no insulin is added to the medium

The index of precision (λ) varies between 0 18 and 0 28 However the results are usually analysed and expressed as the glucose uptake values themselves above the basal level This after all is the essential data from which any estimate of insulin activity can be made

We have been particularly interested in the two broad clinical types of diabetic patient those who require insulin treatment without which they rapidly lose weight and become ketotic and those who do not need insulin and show no tendency to ketosis unless their diabetes is complicated by infection The latter are usually obese and often recover from the diabetic state when they lose weight on a low carbohydrate-reducing diet

Insulin activity was found in the plasma of all these obese patients Also as with normal subjects when insulin was added *in vitro* to the plasma from these patients its activity was not diminished i e there was no measurable insulin antagonism in the plasma This suggests that the well-known resistance to insulin which these patients exhibit resides in the tissues

On the other hand in uncontrolled but non-ketotic diabetic

patients who require insulin no plasma insulin activity was *found* Moreover when insulin was added to the plasma from these patients its activity was apparently inhibited i e not fully recovered If the patients *in the group were controlled so that their blood* was physiological at the time of the test then plasma insulin act was again found essentially in the normal range and now a *insulin could be recovered*[7] *(Fig 1 and Table I)* Again this antag ism could be removed by diluting the plasma prior to assay W the plasma from the uncontrolled insulin-requiring dial diluted 1 in 4 then insulin activity is found (Table II)

It is important to stress that these patients were not from insulin therapy for longer than at the most 24 hours T fore the finding on dilution does not indicate that there is n any endogenous insulin in their plasma although this may It only reveals the activity of the exogenous insulin which h administered to them either 1 hour before the test was or the previous day and is masked by the inhibitor in plasma Also this observation would explain why the using diluted plasma in their assay procedure have ord *to find any significant difference in plasma insulin* normal and diabetic subjects

This antagonism to insulin found in the plasma of *insulin-requiring diabetics may* be an exaggeration of in normal plasma, where the antagonism is completely adequate insulin production by the pancreas In antagonism *can be revealed by pancreatectomy*[8]

Also we have recently found that the antagonism of these patients resides in the albumin fraction of the teins and *that similar antagonism is present in the p* fraction from normal subjects However the al normal subjects is less active in this respect[9]

In this study the freeze-dried fractions obtained in buffer solution containing glucose and insulin ad alone and to the buffer with dissolved fraction to centration of 1000 μu/ml

FRACTIONATION OF DIABETIC PLASMA PROTEINS

As was found previously full saturation of with sodium chloride precipitated the antag plasma[8] The active precipitate did not contain

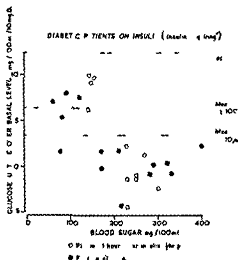


Fig 1 Pl ma insulin activity in ins lin requiring diabetic patients showi between those w th elev ted and physio ogical blood uga

Table I

S MMARY OF EXPERIMENTS O I PLASMA INSULIN ACTIVITY AND INSULIN ANTAG IN DIABETIC PATIENTS

Mean gl cose uptake above the basal level ± st dard error of t e mean (mg /100 ml /10 mg dry wt d phragm)

G ou p	Plasm	Plasm after glucose	Plasm + 400 µ /ml insul n
Obese diabetics	6 97 ± 0 350	9 53 ± 0 99	10 0 ± 0 40
Insulin-requ ring d abetics with elev ted blood sugar	—0 56 ± 0 50		1 10 ± 0 628
Insulin-req iring d abet s with physiological blood s gar	7 24 ± 0 893		9 74 ± 1 697

I the insulin-requiring group with elevated blood sugar whether the pl ma was taken alone or with 400 µ /ml. of insul n added the u take values do not differ significantly from zero—indicating no measurable insul n activity Whereas, in the same group when the blood sugar is physiol gical there is a h ghly significant increased uptake above zero with the pla ma alone and then added insulin can be satisfactorily recovered as is also found with the obese di betic group

contain a substantial amount of albumin Owing to the poor fractionation of the plasma proteins by this method sodium sulphate was employed in subsequent experiments The activity of the fractions obtained by sodium-sulphate fractionation of diabetic plasma is shown in Table III This shows that the precipitate obtained by saturation with 18 per cent sodium sulphate was devoid of antagonistic activity whereas the supernatant had such activity

Paper electrophoresis showed that the sodium-sulphate fractionation effected a complete separation of the γ globulin and albumin fractions i e all the γ globulin was in the precipitate and all the albumin in the supernatant The other globulin components of the

TABLE II

EFFECT OF DILUTING PLASMA FROM INSULIN REQUIRING DIABETICS

When the plasma is undiluted there is no significant glucose uptake above zero indicating no measurable insulin activity whereas when the plasma is diluted 1 4 there is a highly significant increased uptake above zero indicating insulin activity in the plasma

Group and (No)	*Mean glucose uptake above the basal level ± standard error of the mean (mg /100 ml /10 mg diaphragm)*	
	Undiluted plasma	*Plasma /4*
Insulin requiring diabetics with elevated blood sugar (4)	−0 37 ± 0 77	5 47 ± 0 43

plasma were present in both fractions It was concluded that the antagonistic activity was not associated with the γ-globulin fraction

Attempts to fractionate the antagonistic activity of the supernatant from 18 per cent sodium sulphate by higher concentrations of sodium sulphate were not successful (Table III) These results were consistent with the idea that the activity was associated with the albumin in the diabetic plasma Albumin was, therefore prepared from diabetic plasma by the TCA-ethanol method of Debro *et al* [2] As will be seen from Table III this albumin antagonized the effect of insulin The inhibition of the added insulin was complete when the concentration of albumin in the buffer was between 3 5 and 5 5 per cent which includes the physiological range When the concentration was reduced to 1 25 per cent there was still marked antagonism of the added insulin Paper electrophoresis of these albumin fractions failed to reveal any other plasma p

FRACTIONATION OF PROTEINS FROM NORMAL PLASMA

The demonstration of the presence of an insulin albumin from diabetic plasma prompted us to test similar protein fraction from normal plasma As Table IV this normal albumin in physiological c~ had antagonistic activity However unlike albumin plasma a concentration of 1 25 per cent failed to show ism The possibility that this activity of normal plas due to a change in the albumin associated with the i

TABLE III

INSULIN ANTAGONISM OF FRACTIONS FROM DIABETIC PLASMA
(number of observations in parenth ses)

F act on	Me n glu se upt ke b bas l les ± t dard error f th me n (mg /100 ml /10 mg d pl s gm)	
	B ff + 1000 μ /ml ns l	F ct n n b 1000 μμ/ml ms
Precip tate from 18 per cent sodium sulphate	12 70 ± 0 92 (4)	13 17 ± 1 54 (4)
Sup rnatant from 18 per nt sodium sulphate		
a P cipita e from 22 per cent sodium sulph 1	12 68 ± 0 64 (6)	3 02 ± 1 37 (6)
b S pernatant f om 22 per ent sodi m suplate	11 43 ± 1 02 (9)	2 30 ± 1 14 (9)
Album n		
3 3 3 3 per nt	13 22 ± 1 08 (4)	0 37 ± 0 23 (4)
1 25 per cent	12 32 ± 1 05 (4)	1 55 ± 0 67 (4)

method of preparation was tested by examining the activity c two samples of ether-fractionated albumin As shown in the table there also inhibited the action of insulin One sample of ethano' fractionated plasma albumin (Cohn Fraction V) had similar activity The possibility that the antagonistic activity was associated with a substance incapable of precipitation with TCA e g a polysaccharide was excluded by changing the method of preparation of the albumin Thus when the plasma proteins were precipitated with 5 per cent TCA and the albumin extracted from the washed precipitate with ethanol this albumin was fully active

Since the albumin from normal plasma inhibited the action of insulin and yet the whole normal plasma did not exhibit any such antagonism it was of interest to test the activity of fractions obtained from normal plasma by treatment with sodium sulphate It will be seen from Table IV that in contrast with the results with diabetic plasma the precipitates obtained with 18 per cent sodium sulphate stimulated the activity of insulin and this supernatant was

TABLE IV

INSULIN ANTAGONISM OF FRACTIONS FROM NORMAL PLASMA
(number of observations in parentheses)

Fraction	*Mean glucose uptake above basal level ± standard error of the mean (mg /100 ml /10 mg diaphragm)*	
	Buffer + 1000 µu/ml insulin	*Fraction in b ffer +* 1000 µu/ml insul n
Albumin (TCA-ethanol)		
3 5 5 5 per cent	11 43 ± 0 6 (6)	0 50 ± 0 48 (6)
2 5 per cent	12 63 ± 10 4 (3)	6 60 ± 1 01 (3)
1 25 per cent	11 32 ± 0 74 (4)	11 17 ± 0 89 (4)
Albumin (ether fractionation) *5 per cent*	12 50 ± 1 40 (2)	2 75 ± 0 45 (2)
Albumin (Cohn Fraction V) 3 5 per cent	12 40 (1)	2 40 (1)
Precip tate f om 18 per cent sodium sulph te	11 11 ± 0 34 (4)	15 61 ± 0 70 (4)
Supernatant from 18 per cent sodium sulphate	12 42 ± 0 65 (4)	12 15 ± 0 22 (4)

devoid of antagonistic activity Since the amount of albumin in the supernatant would be sufficient to effect complete inhibition of 1000 µu/ml of insulin it appears that normal plasma contains a substance which ... the antag... vity of the albumin This sub... be insulin i...

It seems ... of al... the action of insulin ... gm is ... action on the ... the Firstly

the albumin in these physiological concentrations merely reduced the glucose uptake of the diaphragm to that achieved by the tissue in the absence of added insulin Secondly the addition of large amounts of insulin i e 10 000 µu/ml as above partially counteracted the antagonism of the albumin The results of the experiments illustrating this point are shown in Table V

Very recently we have studied three patients who have been hypophysectomized Two had the pituitary ablated by transnasal implants of yttrium[90] into the sella turcica and one had the pituitary removed by surgery

TABLE V

EFFECT OF THE ADDITION OF 10 000 µU/ML INSULIN TO ALBUMIN FROM NORMAL SUBJECTS

(number of observations in parentheses)

Fraction	Mean glucose uptake above basal level ± standard error of the mean (µg/100 ml/10 mg diaphragm)	
	Buffer + 1000 µu/ml insulin	Fraction in buffer + 10 000 µu/ml insulin
Normal albumin 4 per cent*	12 33 ± 0 93 (4)	5 93 ± 0 67 (4)

* As will be seen from Table IV albumin at this concentration completely antagonize the effect of insulin at 1000 µu/ml

The albumin fraction of the plasma proteins from each of these patients prepared as before was devoid of antagonistic activity[10] (Table VI)

Again diabetic albumin (300 mg) prepared and with the activity previously described was dissolved in 4 ml of borate-phosphate buffer and passed through a column (40 cm × 1 5 cm) of partially acetylated cellulose The protein was eluted with buffer and a total of 150 ml of buffer was collected, the eluent extensively dialysed and the protein freeze dried When the activity of this protein which was electrophoretically identical with albumin was tested, the original activity against insulin was found to have been removed by passage through the column.

These observations would indicate that the antagonism is probably not due to albumin *per se* but to a substance bound to it which is related in some way to the pituitary gland

However the pituitary gland may not be solely responsible for this insulin antagonism as is suggested from studies made on experimental diabetes in animals [8]

Normal depancreatized depancreatized-adrenalectomized (Long Lukens) and depancreatized-hypophysectomized (Houssay) cats have been studied with and without various hormonal replacements

In normal fasting cats the mean plasma insulin activity was 114 μu/ml and when a known amount of insulin was added to the plasma *in vitro* its activity was not diminished

TABLE VI

SUMMARY OF EXPERIMENTS ON ALBUMIN FROM HYPOPHYSECTOMIZED PATIENTS AND ON DIABETIC ALBUMIN PASSED THROUGH A COLUMN OF PARTIALLY ACETYLATED CELLULOSE

Fraction	*Mean glucose uptake above basal level ± standard error of the mean (mg /100 ml /10 mg diaphragm)*	
	Buffer + 1000 μu/ml insulin	*Fraction in buffer + 1000 μu/ml insulin*
Hypox albumin 4 per cent	11 85 ± 0 68 (8)	11 43 ± 0 80 (8)
Diabetic albumin through column 1 25 per cent	13 12 ± 0 65 (6)	12 58 ± 1.28 (6)

In our study of depancreatized cats the animals were handled in one of two ways In one the blood was withdrawn 2 or 3 days after pancreatectomy In the other the depancreatized cats were maintained on adequate amounts of soluble insulin for at least 5 days after operation Insulin injections were discontinued for 3 days before the removal of the blood samples In either case there was no measurable plasma insulin activity in these animals and moreover there was an inhibition of added insulin

This inhibition could be abolished either by hypophysectomy or bilateral adrenalectomy In either Houssay or Long-Lukens cats there was again no plasma insulin activity but now the activity of insulin added to the plasma was not diminished some 100 per cent being recovered

Cortisone or hydrocortisone 10 mg /day injected subcutaneously

for 4 days into the Long Lukens type of animal restored the inhibiting properties of the plasma The same dose of hydrocortisone injected into Houssay cats for 4 days and even longer until severe ketosis was produced did not restore the inhibitory activity of the plasma from these animals

Growth hormone (3 mg /day for 4 days) injected subcutaneously into either Long-Lukens or Houssay cats failed to restore the inhibiting properties to their plasma

TABLE VII

SUMMARY OF EXPERIMENTS ON PLASMA INSULIN ACTIVITY AND INSULIN ANTAGONISM IN CATS

Gro p a d (N)	*Mean gl pt ke bove the level of d ph gm b ffer lo e ± st dard error of the mean (mg /100 ml /10 mg d aphragm)*		
	*Buffer + insul n**	*Pl ma lo e*	*Plasma + sul **
Norm l (7)	12 63 ± 0 74	6 60 ± 0 73	13 33 ± 0 82
D p creatized (9)	11 73 ± 0 50	0 57 ± 0 48	6 19 ± 0 47
Dep hyp x (3)	11 60 ± 0 68	0 0 ± 0 09	11 10 ± 0 75
D pan hypox + F (3)	11 55 ± 0 74	0 14 ± 0 32	12 25 ± 0 63
D pan hypox + GH (4)	11 33 ± 0 35	0 8 ± 0 52	11 95 ± 1 05
Dep d ex. (3)	12 53 ± 0 27	0 81 ± 1 03	13 22 ± 0 81
Depan ad ex + E or F (3)	11 52 ± 0 64	—0 50 ± 0 89	2 57 ± 1 92
D p n. adrex + GH (3)	11 80 ± 0 79	—0 50 ± 0 58	11 60 ± 0 82

* In ulin was added to make 1000 micro-units/ml of buffer or pl ma
In the n rm l nin al there is a highly s gnificantly increased uptak f gl ose bove zero b t in all the other oper ted ani al in whi h pancreatectomy w the common feature the gluc se ptake does not differ s gnificantly from r indicating no m surabl insulin ctivity The no signif t diffe en between the glucose uptak in buffer pl s insulin and plasma plus insulin, ex ept the depa creatized and th d p ncreatized adrenalectomized (Long Luken) animal treated with cortisone or hyd o tisone In these two gro p ther is highly significa t decrea of gl ose pt ke with plasm samples s comp red with buffer indicati g the inhib ti of sul n activity

Table VII summarizes the results which suggest that both the pituitary gland and the adrenal oxysteroids must be present for insulin antagonism to be found in the plasma of depancreatized cats

Field and Stetten have been studying the acute insulin resistance found in severe diabetic acidosis[3] Using the Stadie dipping technique and measuring the glycogen deposition in the rat diaphragm they have found considerable insulin inhibitory activity in the plasma of such patients It is evanescent often disappearing within a few hours or at least in a day or so of the commencement of insulin therapy

This activity has been found to reside in ʳ of the plasma proteins and it is not a L
This α_1-globulin antagonist is probably ⁿ that we have described

Turning to studies made with ^{131}I-la New York[1] and Williams in Seattle active in this field[11]

It has been found that in diabetic patients phrenics who have been treated with insulin the disappearance of labelled insulin from p than in subjects who have never been treated persistence of this relatively high concentration is due to binding by a factor in the plasma satisfies the criteria of an antibody and resides γ-globulin fractions This factor in the plasma subjects so binds labelled insulin that it hinders its attachment to rat diaphragm and depresses its by liver slices Also bio-assay studies show that added to this binding diabetic plasma there is som its usual hypoglycaemic effect in mice

It is difficult to assess the importance of this diabetes for there was no real correlation between quirements of the diabetics studied and the degree c labelled insulin in their plasma Indeed about one-fifth treated with insulin did not exhibit any increased

Also since the majority of insulin-treated rem sive to exogenous insulin it must be supposed that antibody complex is usually readily dissociable or that it the pharmacological activity of native insulin

This antagonist is clearly different from the one we have in the albumin fraction of the plasma proteins

Conclusions

Uncontrolled insulin-requiring diabetics have no plasma insulin activity and when insulin is added to their its activity is inhibited

If these patients are controlled however plasma insulin is found essentially in the normal range and added insulin can be recovered

The insulin antagonism in these patients has been found to reside in the albumin fraction of the plasma proteins and is also present to a lesser extent in the albumin fractions of the plasma from normal subjects It is not due to the albumin itself but to a substance bound to it related in some way to the pituitary gland

This antagonist appears to be different from those described by other authors

Finally as we were told by Professor Young to let our hair down I would like to suggest that whatever the site and mode of action of insulin in carbohydrate metabolism it has first to overcome the antagonistic activity of this substance attached to albumin Perhaps the considerable confusion surrounding the question of insulin action on the liver may be due to this fact remembering that the liver has more than its fair share of albumin

REFERENCES

1 BERSON F A YALOW R S BAUMAN A ROTHSCHILD M A & NEWERLEY K (1956) *J Cl I t* 35 170
2 DEBRO J R TARVER H & KORNER A (1957) *J Lab Cl n Med* 50 728
3 FIELD J B TIETZE, F & STETTEN D JNR (1957) *J Cl I vest* 36 1588
4 GROEN J KAMMINGA C E WILLEBRANDS A F & BLICKMAN J R (1952) *J I I t* 31 97
5 RANDLE, P J (1954) *B t med J* i 1237
6 VALLANCE-OWEN J & HURLOCK B (1954) *La et* i 68
7 VALLANCE-OWEN J HURLOCK B and PLEASE, N W (1955) *Lancet* ii 583
8 VALLANCE-OWEN J & LUKENS F D W (1957) *Endocri l gy* 60 625
9 VALLANCE-OWEN J DENNES E & CAMPBELL P N (1958) *La t* ii 336
10 VALLANCE-OWEN J DENNES E & CAMPBELL P N (1958) *Lanc t* ii 696
11 WELSH G W III HENLEY E D WILLIAMS R H. & ELGEE N J (1956) *D abete* 5 15

ANTIBODIES TO INSULIN

P J MOLONEY

Connaught Medical Research Laboratories University of Toronto

There is a question which might be asked even at this late date and which is suggested by the subject for discussion and the question is this are antibodies induced by insulin? It is true that an affirmative answer has already been given to the question still there are some reasons why it might be re-examined For example there is good evidence to indicate that certain *in vitro* reactions which have been ascribed to insulin-anti-insulin are due instead to antigen other than insulin which may contaminate crystalline insulin and corresponding antibody[5] There is also the matter of inactivation of insulin by serum from insulin resistant animals One might ask is it possible that such neutralization is effected not by antibody but by destruction of insulin as for example by an enzyme?

I shall have something to say about these questions and I hope to show that insulin can indeed induce in animals a corresponding antibody and in addition I shall present results which have to do with different types of anti-insulins and with insulins which differ in respect of neutralization

By antibodies to insulin I mean antibodies which neutralize the physiological action of insulin This is the type of antibody which is encountered with certain diabetics fortunately rarely and which is responsible in these for the ineffectiveness of insulin to control the disease

Resistance to insulin thanks to antibody formation can also be induced in animals as was first shown by Lowell and Franklin[6] with the rabbit

In our work we induced antibody formation in animals of a number of species by the injection of crystalline ox or pig insulin in Freund s adjuvant

The presence of antibodies in the sera of animals and of humans was detected by a method first used by Banting *et al* [1] namely inhibition by antisera of insulin induced hypoglycaemic convulsions in mice In detail starved mice were injected with serum or

dilutions of serum mixed with an amount of insulin which when injected with saline or with normal serum caused convulsions in 90-100 per cent of the mice. Table I gives results of a typical test.

Three groups of mice were injected as shown. Each mouse received the same amount of insulin, namely 0.05 unit. Each mouse

TABLE I

MOUSE NEUTRALIZATION TESTS

Mixture injected per mouse			*Number mice injected*	*Number convulsions*	*Comment*
Units insulin		*Volume and dilutions of serum*			
0.05	+	0.25 ml. undil.	6	0	Insulin neutralized
0.05	+	0.25 ml. 1/2 dil	6	6	Insulin not neutralized
0.05	+	0.25 ml 1/4 dil	6	6	Insulin not neutralized

received the same volume of serum or of diluted serum, viz 0.25 ml. *The mice which received undiluted serum showed no convulsions*, all other mice showed typical convulsions. That is 1 ml of undiluted serum was capable of neutralizing 0.2 unit of insulin but was not capable of neutralizing 0.4 unit.

TABLE II

NEUTRALIZATION OF INSULIN WITH ANTISERA

Anti-insulin (origin)		*Insulin (extracted from pancreas of)*
Guinea pig Rabbit Sheep Horse Human	Neutralizes	Pig (Swine) Ox Rabbit Sheep Horse Monkey (macaca rhesus) Chicken Fish (Ling) Whale Human

Table II gives a summary of some neutralization results.

Anti-insulin had been induced in the guinea-pig, the rabbit, the sheep and the horse by a series of injections of crystalline ox or pig insulin in Freund's adjuvant. The human serum was from a diabetic who had developed resistance to insulin.

The neutralizing capacity of each serum was tested against each of the extracted insulins shown in the second column by the convulsion-inhibition test It was found that each antiserum neutralized each of the insulins shown That is antiserum from the guinea-pig neutralized not only pig or ox insulin which had been used to induce anti-insulin but also insulin from the rabbit sheep horse monkey chicken fish (ling) whale and human and so with antiserum from the rabbit and the sheep and the horse and the human And note that antiserum from the rabbit neutralized insulin extracted from rabbit pancreas and similarly antiserum from the sheep the horse and the human neutralized insulin extracted from the pancreas of the sheep the horse and the human respectively

There were good reasons for the assumption that neutralization of insulin by antiserum was an antigen-antibody phenomenon For example just as with antibodies in general anti-insulin had been induced by a series of injections of a protein in this case insulin neutralizing activity was associated with the globulin fraction of the antiserum and finally insulin could be recovered from a neutral insulin-anti-insulin mixture by extraction with acid alcohol thus indicating that inactivation was not due to destruction

Since it appeared probable that one had to do here with an antigen-antibody system the possibility of *in vitro* reaction was explored and in particular the possibility of precipitation or flocculation in mixtures of insulin and anti insulin In all preliminary observations which were made using anti insulin derived from the guinea-pig rabbit sheep and horse precipitation was not observed Finally however in mixtures of insulin and certain specimens of serum from a resistant horse typical flocculation occurred

A summary of the course of immunization of the horse which yielded flocculating serum is given in Table III

Lots A B C and D consisted of relatively large amounts of serum 1 litre or more Flocculation occurred with mixtures of insulin and serum of lots C and D but not with serum of lots A and B

I shall give very briefly results on flocculation from a paper which has been submitted for publication [8]

Results in Table IV have to do with the determination of the equivalence zone of insulin-anti insulin

Each flocculation mixture consisted of a constant amount of flocculating serum (0 2 ml) plus the amount of insulin shown in the first column in a total volume of 0 7 ml pH 7 4 Equivalence zone

was determined by standard procedure namely two sets of mixtures as in the first two columns were prepared After flocculation had taken place all mixtures were centrifuged and supernatants poured off To one set of supernatants a constant amount of anti insulin as shown was added and to the other set a constant amount

TABLE III

RESPONSE OF HORSE NO 2589 TO INJECTIONS OF INSULIN

Time (days)	Units of insulin injected	Potency of serum (units insulin neutralized per ml serum)
0	6 50	
28	6 50	
33		0 4
47		0 2
49	6250	
61		0 8 Lot A
70	6250	0 8 Lot B
85		0 8
92	6250	0 8
105		0 8 Lot C
112	6 50	0 8
134		0 8 Lot D

of insulin Flocculation results with these are shown in the last two columns The equivalence zone is that in which there is excess of neither insulin nor anti-insulin

From washed floccules of the equivalence zone we were able to recover insulin and anti-insulin in good yields The recovered insulin and anti-insulin were assayed by mouse convulsion technique

TABLE IV

EQUIVALENCE ZONE OF INSULIN ANTI INSULIN

Units of insulin in 0 5 ml	Antiserum C ml	Flocculation	Flocculation in supernatants	
			Plus 0 025 ml antiserum C	Plus 0 25 units insulin
0 46	0 2	+	−	+
0 58	0 2	+	−	+
0 71	0 2	+	−	+
0 89	0 2	+	−	tr
1 0	0 2	+ 1st	−	−
1 2	0 2	+ 1st	+	−
1 4	0 2	+	+	−
1 7	0 2	+	+	−
2 1	0 -	+	+	−

and not by flocculation Here then is evidence that typical antigen-antibody flocculation may occur with insulin and anti-insulin

This conclusion is supported by other evidence

Table V shows graphically the result of an agar diffusion test

TABLE VI

EFFECT OF ANTI INSULIN ON ENDOGENOUS INSULIN

A ti-ns l n (or g)		*E doge ous nsul n*
Guin a p g	Neutr liz s	Mouse
Hors	Does not neutraliz	Mouse
H m n	Does not neutraliz	M use
Mouse Guinea p g R bb t Sh ep Ho se Human	Endog s insulin of anti insuli produ er n t neutralized	

The insulin and anti-insulin solutions were equal in volume and were in equivalence amounts The position and curvature of the line of precipitation is consistent with a smaller molecular species insulin diffusing towards a larger molecular species anti-insulin (horse globulin)

There is a *further piece of evidence* which indirectly supports the conclusion that flocculation is due to insulin and anti-insulin and which depends on the following considerations Boyd and Hooker[9] derived a formula for the relation between the ratio of antibody to antigen in floccules at the equivalence point and the molecular weight of antigen

The ratio of anti-insulin to insulin in the equivalence zone was calculated from total nitrogen of floccules and an estimate of th amount of insulin precipitated

When this ratio was used in the formula of Boyd and Hooker a value for the molecular weight of insulin of 24,000 to 36 000 was obtained. This value is in the order of magnitude of the molecular weight of insulin in aqueous solution pH 7 to 8

There are different types of neutralizing antibodies These for example can be flocculating or non-flocculating Indeed the flocculating serum with which we worked contained in addition to flocculating antibodies non-flocculating antibodies as we were able to show by fractionation with ammonium sulphate Other differences in antisera are shown in Table VI Here are three neutralizing antisera viz guinea-pig horse and human all of which were produced by injection of crystalline ox or pig insulin Only the antiserum from the guinea-pig neutralized mouse-endogenous insulin as indicated by the production of signs of diabetes in the mouse In this connection some work of Lacy and Davies[4] is of interest These investigators labelled with fluorescein isocyanate a globulin fraction of guinea-pig serum containing anti-insulin The labelled anti-insulin globulin was used to stain frozen sections of pancreas from beef mouse guinea-pig rabbit and man A yellow green fluorescence was produced in the islets of mouse and ox pancreas which was inhibited by prior addition to the antiserum of crystalline pork insulin Fluorescence was not produced in the islets of guinea-pig rabbit or human pancreas Results with mouse and guinea-pig pancreas and these are the only two for which we have relevant data are consistent with our findings, namely that antiserum of the guinea-pig neutralized endogenous mouse insulin but did not neutralize endogenous guinea-pig insulin[4]

Also in Table VI there are indicated differences in insulins in respect of neutralization The endogenous insulin of the anti-insulin producer is not neutralized Whereas for example anti-insulin from the horse neutralized insulin extracted from *horse*

pancreas it did not neutralize the endogenous insulin of the horse-producing anti insulin since the animal was healthy and showed no signs of diabetes and so for the antisera of the rabbit and the sheep and even the human This latter was a severe diabetic woman whose diabetes could not be controlled by insulin nor by an oral preparation The disease however was controlled by a low caloric diet and on this regimen the patient successfully withstood the amputation of a leg which had to be done because of a gangrenous foot.[3]

Table VII is also concerned with differences in insulins This table gives a summary of properties of guinea-pig insulin

TABLE VII

GUINEA PIG INSULIN

1	Mixture of guinea p g and ox insulins may be separated by partition chrom togr phy
2	Guinea-pig insulin not ne trali ed by anti-insulin (mouse con ul ion te t)
3	Guinea p g insulin low rs blood g of mouse inj ted with anti-insuli f om gui p g
4	Gui ea-pig in ulin *d e t* lower bl d sugar of anti sulin prod cing guine p g

Since guinea-pig insulin is not effective in lowering the blood sugar of a guinea pig in which antibodies have been induced by ox or pig insulin it is altogether improbable that it would be useful for the treatment of a diabetic who has developed antibody-type resistance to injected insulin

Table VIII shows a comparison of neutralization of ox and cod insulins

TABLE VIII

COMPARISON OF COD AND OX INSULINS

Anti-insul n (*hor e*)	*Fract co vul g*	*A ti- sul n* (*l se*)	*F act vuls g*
	Ox in l (0 05 *u per n o s*)		*Cod insul* (*pp* x 0 03 *u per mo se*)
Undil	0/12	U dil.	2/12
1/2	0/12	1/2	7/12
1/4	1/12	1/4	8/12
1/8	12/12	1/8	7/12
Saline	12/12	Saline	8/12

These are different Evidence from flocculation and neutralization[8] indicates that soluble ox insulin-anti-insulin complex is dissociated Results here indicate that soluble cod insulin-anti insulin complex has a larger dissociation constant than ox insulin anti-insulin

It would be interesting to know why endogenous insulins are not neutralized by antibodies which neutralize insulin extracted from pancreas of the same species And it would certainly be desirable to have available an insulin effective in insulin-resistant diabetics that is to say an insulin which is not neutralized by antibodies *induced in the human by* ox or pig insulin

Summary

By the injection of ox or pig insulin into animals it is possible to *induce the formation of antisera which are capable of neutralizing* the physiological effect of insulins extracted from the pancreas of a number of species of animals Such sera in mixture with insulin may or may not exhibit the phenomenon of flocculation A study of flocculation and neutralization with an anti-insulin serum from a horse showed clearly that neutralization of insulin by the serum was an antigen-antibody reaction It is highly probable that neutralization of insulin by non-flocculating antisera is also an antigen-antibody reaction

Antibodies to insulin may differ not only in respect of flocculation and non-flocculation but also in respect of insulins neutralized For example antiserum induced in the guinea-pig by the injection of ox or pig insulin was capable of neutralizing endogenous mouse insulin *whereas anti-insulins induced in the horse human* and mouse were not

Extracted insulins from the pancreas of the ox pig rabbit sheep horse monkey chicken fish (ling) and man were neutralized by antisera induced by ox or pig insulin in the guinea-pig rabbit sheep horse and man Extracted insulin from guinea-pig pancreas was not neutralized as shown by inhibition of convulsions and by blood-sugar test in mice it was however neutralized in the guinea pig carrying antibodies actively induced by ox *or pig insulin* Insulin extracted from cod pancreas is incompletely neutralized an effect which depends probably on mass action

REFERENCES

1 BANTING F G FRANKS W R. & GAIRNS S (1938) *Amer J Physch at* 95 562
2 BOYD W C & HOOKER, S B (1938 39) *J Gener l Phys ol* 22 281
3 EZRIN C. & MOLONEY P J (1959) *J Cl D d c Met b* 19 1055
4 LACY P E. & DAVIES J (1957) *Diab tes* 6 354
5 LAPRESLE, CL & GRABAR P (1957) *Re e Franç ise d'Ét d Cl ques et B ol g ques* 2 1025
6 LOWELL F C & FRANKLIN W (1949) *J Cl n Invest* 28 199
7 MOLONEY P J & COVAL M. (1955) *B ochem J* 59 179
8 MOLONEY P J & APRILE M. A (1959) *Can J B ochem Phys l* 37 7B

DISCUSSION

YOUNG Dr Moloney has introduced into the subject which we are considering during these two days the ideas and methods of investigation of the immunologist and the serologist in a most important and I think entertaining fashion

The particular interest pertaining to guinea pig s insulin is one that I certainly share More than ⌐0 years ago H P Marks and I (*Nature* 1940 146 31) prepared a small quantity of insulin from guinea-pig pancreas We were very interested in the relatively low yields of insulin which we obtained from the pancreas of this animal The yield per 100 g of body weight was less than one tenth of that for the other species which we were investigating at the time. This was unexpected in view of the fact that in the guinea pig pancreas there are clear granules in the β cells of the pancreatic islets which diminish in number when the blood sugar rises and which show many signs of being the precursors of insulin It is possible that there is an antagonistic factor to guinea pig insulin in the pancreas itself though Griffiths who was in the laboratory with us got some evidence for this later we were not able clearly to demonstrate this Perhaps Dr Moloney would care to comment on these observations

With regard to the species differences of insulin the work of Sanger – which has covered a considerable number of different insulins has shown chemical differences in the amino acids within the intra-chain disulphide link of the A chain of insulin although as far as I know nothing is known about the structure of guinea pig insulin

I was particularly glad that Dr Vallance-Owen still thinks that the pituitary gland is a going concern It is a concern in which I myself have been interested for many years and I am still unrepentant in my belief that growth hormone is of particular importance in the antagonism to insulin which is exerted by pituitary extracts and by the secretions of the pituitary gland itself

I noted that Dr Vallance Owen in his experiments with Dr Lukens found no clear effect of growth hormone in the Houssay animal when the growth hormone was given alone I am not clear whether growth hormone and a small amount of adrenal steroid were also given in similar experiments Dr Vallance-Owen, was that so?

VALLANCE OWEN No Sir Growth hormone was given alone to the Houssay animals adrenal steroids were not given in addition

YOUNG Do you think the growth hormone is concerned in the pituitary effect that you and Dr Lukens observed?

VALLANCE-OWEN Yes I do

YOUNG I am glad about that One might ask now what is the physiological significance of this antagonism between pituitary hormones on the one hand of which I think growth hormone is one and insulin on the other? This question is an important one because there is some evidence which I cannot discuss now that in some species of animals an increased secretion of insulin by the islets of Langerhans of the pancreas is stimulated by growth hormone Why one may ask should there be a stimulation of insulin secretion by the pancreas in some species at the same time as there is produced in these animals by means which are not certain and by a mechanism which is still far from clear an antagonism to the action of insulin as far as carbohydrate utilization in muscles is concerned?

I do not think any final answer can be given to this question but I still hold to the view that I have held now for quite a few years of the importance of insulin in protein anabolism The antagonistic action of the growth hormone or the insulin antagonist it elicits the effect of insulin on carbohydrate utilization may permit the effectiveness of insulin on protein anabolism to be emphasized without a danger of an undesirable fall of blood-sugar level

The variety of known antagonists to insulin is becoming rather disturbing and we cannot assume at the present time that there is any one main antagonist to the action of insulin on carbohydrate utilization There may exist a series of antagonists existing in different species under different conditions but much research needs to be done on this point at the present time

The question of the presence of insulin in blood plasma masked by antagonists is one that Dr Randle and I met some years ago when we found that in a cat which had been rendered persistently diabetic by pituitary treatment the metahypophyseal diabetes was associated with a condition in which no insulin could be detected in the plasma by the rat diaphragm method as used in our laboratory If the plasma of this diabetic animal was frozen and thawed three times and the insulin activity then measured by the diaphragm method insulin activity was found to have appeared and to be about the normal amount Is it possible therefore that in this animal with diabetes originally of pituitary origin but associated more directly and primarily with islet lesions there is insulin circulating in a form which is completely neutralized as far as action on the normal diaphragm method is concerned but which can be

unmasked by a process which might be expected to inactivate a lipoprotein? I do not know what the final answer to that question is but it again emphasizes the dangers of measuring insulin in plasma by only one method and of making any assumption that one is in fact measuring insulin itself

Some experiments carried out by Dr Whitney and myself (*Biochem J* 1957 66 648) some years ago are possibly relevant to this point We investigated the effect of the intravenous injection of glucose (.00 mg /ml) on the plasma insulin activity of the normal rat, and we also measured the uptake of glucose by the diaphragm of these rats given glucose intravenously Control experiments were carried out with diaphragm from rats which were not treated with glucose

We found as we rather expected, that when the blood sugar level had been raised by the injection of glucose the capacity of the diaphragm of the injected animal to take up glucose when the diaphragm was excised and immersed in glucose-containing buffer also rose returning to a little below the normal at about 120 minutes

We took the plasma from these glucose-injected rats and assayed it by the diaphragm method for insulin like activity We rather expected that the plasma insulin activity would be raised soon after the injection of glucose but in fact we found that it was very definitely lowered rising to about the normal level at 60 minutes after which it rose above normal where it remained until the end of the experimental period of 1.0 minutes

I cannot believe that insulin secretion by the pancreas is not increased when the blood-sugar level rises There is too much evidence of all sorts to allow a contrary view to be accepted It is possible that insulin which was present in the plasma to begin with became bound in the tissues when the blood sugar level rose and there may therefore have been a removal of insulin into the tissues at such a rate that insulin replacement by secretion from the pancreas was initially unable to maintain a steady level of blood insulin There is also the possibility that insulin antagonists were secreted into the blood stream soon after glucose had been injected but I do not know what is the simple explanation of these findings This type of experiment emphasizes the complexity of the situation which one is dealing with in discussing the activity of insulin in plasma and it also shows that the secretion or development of antagonists to insulin might occur under physiological conditions for reasons which I think we cannot easily understand at the present time

I personally have very much enjoyed the papers today Particularly that in which Dr Moloney made so clear the immunological properties of the system he has investigated The fact that his antibodies to insulin provide a valuable tool for the elucidation of the problems that we have been discussing has already been clearly illustrated by the results which

Dr Taylor at Cambridge and Dr Wright in London put before us earlie this morning Undoubtedly Dr Moloney has placed an extremely valuable and powerful tool in the hands of those who are interested in these problems

PETROW It has been shown in a limited number of publications that growth hormone appears to stimulate resistance to infection of animals through some adrenal mechanism I just wondered whether to the extent to which antibodies are involved in that effect you might not have a partial explanation of growth hormone antagonism to insulin through an antibody mechanism of this sort

YOUNG Dr Petrow I presume you are referring to Selye s work here I do not know that there is any clear evidence of the interpretation which you suggest It is a possible one but I prefer to reserve judgment on this aspect of the problem

The effects of growth hormone on the adrenal cortex of the hypophysectomized rat were investigated by Stack Dunne *et al* (*J Endocrinol* 1955 **12,** 174) in my laboratory some years ago There is no clear indication that growth hormone stimulates the secretion of adrenal steroids There is some stimulation of mitosis in the adrenal cortex but I am very hesitant about the interpretation of much of this work at the present time

FISHER I do not think that in view of the complexity of the situation that has been disclosed here that the injection of another possible complexity can do much harm However one thing which occurs to me about a great deal of this assay work and the effect of protein fractions is that the assumption seems to be inherent that the diaphragm itself is invariant This sort of effect and some of the stimulant effects which Dr Vallance-Owen has described could be the result of non specific or other specific effects of serum proteins on the diaphragm metabolism

I am reminded of some work which Krease has just published showing that the diaphragm however carefully prepared tends to lose potassium fairly rapidly and that the only way he can prevent that is by the addition of various serum protein fractions It has been known for a long time — D K Hill was the first person to do it — that an increase in potassium concentration extracellularly in muscle has a very profound effect on this metabolism

These are considerations which might be borne in mind before one is quite sure that these effects are specific insulin-stimulating or insulin inhibiting

STEWART May I just say something about the point which Professor Young has brought up on the insulin content of the guinea pig pancreas? We have devised a technique for measuring the insulin content of acid alcohol extracts using the mouse convulsion method If we feed mice on a carbohydrate diet and pre-treat them in the fasted state with Phenergan

they are extremely sensitive to insulin. We find that using this technique the guinea pig has between 1-1½ units per gramme fresh weight of pancreas compared with 2-5 units per gramme for the rabbit so the difference is not all that great, and our values are much higher than those found in the literature We think it is something to do with the actual working up of the insulin from the pancreas of the guinea pig which may be quite different from that of working up insulin from ox pancreas

OAKLEY *With regard* to the detection of insulin antibodies Dr Cunliffe has been using a test at King s which is as yet unpublished in this connection but in our hands it does seem to give so far encouraging results What he has been doing is a passive cutaneous anaphylaxis test which consists of giving intradermal injections into a guinea pig of the serum which one is investigating – these of course can be done in varying dilutions – and 24 hours later injecting into one hind leg 1 ml of pontamine blue and 80 units of crystalline soluble insulin into the other *hind leg* If insulin antibodies were present you would get a rapid formation of a circumscribed *blue* ring or area around the site of injection of the serum containing the antibody

It is a relatively simple test in our hands which has so far given remarkably consistent results although we have not been using it long enough However in thirty or forty controls they have all been negative and in thirty or forty diabetics not on insulin there has not been a single positive In large series of diabetics on insulin we have had two weak positives *in* patients whose insulin requirements have been just about 100 units of soluble insulin a *day* and with eleven patients whose insulin requirements have been between 500 and ,000 units of insulin a day we have had a strong positive result in every case

SCHAMBYE I just want to ask Dr Vallance-Owen and Dr Taylor if they would like to comment on the fact that in Dr Taylor s experiments where he used labelled insulin he found all the insulin in the last part of the albumin fraction in the α and α_1-globulin fraction Is it possible that the albumin factor of Dr Vallance-Owen plays any role in the determination *of the* insulin activity in the fraction which Dr Taylor studied?

WRIGHT I would like to ask Dr Moloney whether he has tested any insulin antiserum produced in rabbits by Drs Arquilla and Stavitsky have tested the action of a specimen of such serum in high conc...
upon the effects of insulin on the isolated rat diaphragm and have fou
that even in high concentration it has no effect

MOLONEY Anti insulin produced in the rabbit by Stavitsky Arquillo and in the guinea pig and sheep by us were assayed in Clevela by the Stavitsky and Arquillo *in vitro* method and in Toronto by mo convulsion inhibition method Results in Cleveland were rabbit ser

effective at 1/64 dilution guinea pig serum at serum at 1/4 results in Toronto were g ... vented convulsions at 1/2 dilution rabbit serum action that is to say the results of the assays were

TAYLOR In answer to Dr Schambye may I say our albumin by fractional precipitation, insulin with it As I understand it Dr Vallance-Owen al be present in some of his albumin fractions of course with his insulin antagonist Effects on then very likely represent our balance between insu both present together in the albumin fraction

PART VIII

MECHANISM OF ACTION OF INSULIN AND OTHER HYPOGLYCAEMIC SUBSTANCES

Chairman PROFESSOR C. DE DUVE

THE PERIPHERAL UPTAKE OF C I· RELATIONSHIP TO THE MODE OF AC· AND OTHER HYPOGLYCAEMIC SI

F W WOLFF M HARRISON and G A

The Wellcome Foundation and The Post Grad· London

This work was undertaken to discover wh ponsive to tolbutamide possess a glucose utilization any way from non-diabetics on the one hand and moderate to low doses of insulin on the other The not respond to tolbutamide The absence of ke observed in those diabetics responsive to sulphony that their own pancreas secreted sufficient insulin to We considered that a positive increase of a capillary after the oral administration of glucose would presence of endogenous insulin

The procedure adopted was as follows all subjects a 200 g carbohydrate diet for 7 days prior to the test been stabilized on tolbutamide this was replaced by p the week prior to the test The injection of insulin in normally required it was stopped 36 hours prior to th subjects fasted overnight On arrival they were kept for in a quiet temperature-controlled room Capillary blood specimens were obtained in heparinized tubes as Fig 1

A single stab into the tip of the index finger with a Ba scalpel ensured free flow of blood throughout the Venous blood samples were obtained without the use of a to from the same vein in the median cubital fossa

The blood-sugar concentrations were determined in quadr by the anthrone method The standard error of a single det tion was ± 1 56 per cent. Capillary blood samples were 5 minutes prior to and after the obtaining of the venous The mean of these two capillary blood sugar concentrations used as an estimate of the capillary blood sugar at the time the

sample was obtained Twenty minutes after the commencement of the test 30 g of glucose per square metre body surface were then administered in 200 c c of lemon-flavoured tap water and the test then continued for another hour

Persons attending a diabetic clinic were divided into four groups Group one contained the good responders to tolbutamide Those in group two were termed moderate responders to tolbutamide – they were those patients who showed initial response but later on a partial relapse Group three contained the non-responders to tolbutamide who however never showed ketosis but who required

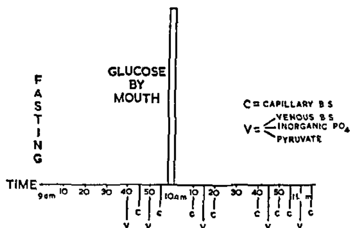


FIG 1 Glucose utilization test

small to moderate amounts of insulin Diabetics with a history of ketosis who required insulin constituted the fourth group A fifth group of non-diabetic subjects was included in the trial to serve as controls

The mean capillary venous difference prior to glucose administration was subtracted from the mean value of the differences estimated during 1 hour after glucose administration This final value was taken to be the measure of one of the effects of endogenous insulin Fig 2 shows the results of these tests in the five groups of subjects Of the diabetics the group responding to tolbutamide showed the best uptake after glucose but even so this was only approximately one-half that of the group of non-diabetic controls

There was no significant difference between moderate responders and non-ketotic non-responders to tolbutamide but the difference between non ketotic non responders and good responders was significant. The ketotic diabetics showed a negative uptake Among those non-diabetics initially chosen for the test were two with disseminated sclerosis two others with presumptive cortical or pituitary dysfunction These are shown in Fig 3 and are characterized by showing a negative uptake

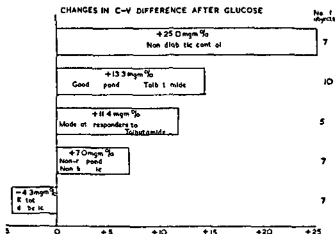


FIG 2

It might be considered that the rise of capillary blood sugar following the administration of glucose was a function of the metabolic abnormality Fig 4 shows a plot of the maximum capillary blood sugar following the administration of glucose plotted against the initial capillary blood-sugar concentration It can be seen that the maximum capillary blood sugar level was correlated with the initial capillary blood-sugar concentration and was independent of the type of diabetes under examination The figure also shows the narrowness of dispersion of the different groups along the regression line

sample was obtained Twenty minutes after the commencement of the test 30 g of glucose per square metre body surface were then administered in 200 c c of lemon-flavoured tap water and the test then continued for another hour

Persons attending a diabetic clinic were divided into four groups Group one contained the good responders to tolbutamide *Those in group two were termed moderate responders to tolbutamide* – they were those patients who showed initial response but later on a partial relapse Group three contained the non-responders to tolbutamide who however never showed ketosis but who required

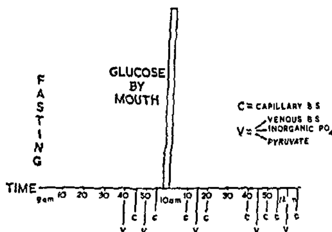


FIG 1 Glucose utilization test

small to moderate amounts of insulin Diabetics with a history of ketosis who required insulin constituted the fourth group A fifth group of non-diabetic subjects was included in the trial to serve as *controls*

The mean capillary venous difference prior to glucose administration was subtracted from the mean value of the differences estimated *during 1 hour* after glucose administration This final value was taken *to be the measure of one of the effects* of endogenous insulin. Fig 2 shows the results of these tests in the five groups of subjects Of the diabetics the group responding to tolbutamide showed the best uptake after glucose but *even* so this was only approximately one-half that of the group of non-diabetic controls

There was no significant difference between moderate responders and non-ketotic non-responders to tolbutamide but the difference between non-ketotic non-responders and good responders was significant The ketotic diabetics showed a negative uptake Among those non-diabetics initially chosen for the test were two with disseminated sclerosis two others with presumptive cortical or pituitary dysfunction These are shown in Fig 3 and are characterized by showing a negative uptake

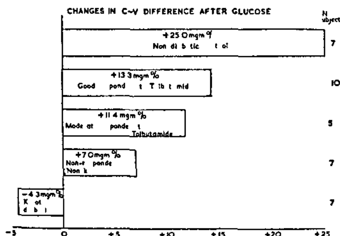


Fig 2

It might be considered that the rise of capillary blood sugar following the administration of glucose was a function of the metabolic abnormality Fig 4 shows a plot of the maximum capillary blood sugar following the administration of glucose plotted against the initial capillary blood-sugar concentration It can be seen that the maximum capillary blood-sugar level was correlated with the initial capillary blood-sugar concentration and was independent of the type of diabetes under examination The figure also shows the narrowness of dispersion of the different groups along the regression line

sample was obtained Twenty minutes after the commencement of the test 30 g of glucose per square metre body surface were then administered in 200 c c of lemon-flavoured tap water and the test then continued for another hour

Persons attending a diabetic clinic were divided into four groups Group one contained the good responders to tolbutamide Those in group two were termed moderate responders to tolbutamide – they were those patients who showed initial response but later on a partial relapse Group three contained the non-responders to tolbutamide who, however never showed ketosis but who required

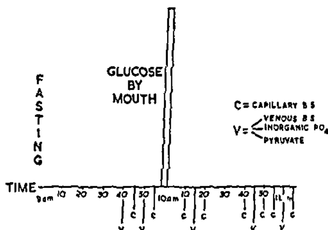


Fig 1 Glucose utilization test

small to moderate amounts of insulin Diabetics with a history of ketosis who required insulin constituted the fourth group A fifth group of *non-diabetic subjects* was included in the trial to serve as controls

The mean capillary venous difference *prior* to glucose administration was subtracted *from* the mean value of the differences estimated during 1 hour after glucose administration This final value was taken to be the measure of one of the effects of endogenous insulin *Fig 2* shows the results of these tests in the five groups of subjects Of the diabetics the group responding to tolbutamide showed the best uptake after *glucose* but *even so* this was only approximately one-half that of the group of non-diabetic controls

There was no significant difference between moderate responders and non-ketotic non-responders to tolbutamide but the difference between non-ketotic non-responders and good responders was significant The ketotic diabetics showed a negative uptake Among those non-diabetics initially chosen for the test were two with disseminated sclerosis two others with presumptive cortical or pituitary dysfunction These are shown in Fig 3 and are characterized by showing a negative uptake

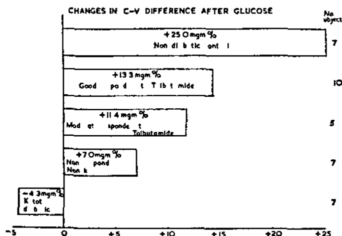


Fig 2

It might be considered that the rise of capillary blood sugar following the administration of glucose was a function of the metabolic abnormality Fig 4 shows a plot of the maximum capillary blood sugar following the administration of glucose plotted against the initial capillary blood-sugar concentration It can be seen that the maximum capillary blood-sugar level was correlated with the initial capillary blood sugar concentration and was independent of the type of diabetes under examination The figure also shows the narrowness of dispersion of the different groups along the regression line

sample was obtained Twenty minutes after the commencement of the test 30 g of glucose per square metre body surface were then administered in 200 c c of lemon-flavoured tap water and the test then continued for another hour

Persons attending a diabetic clinic were divided into four groups Group one contained the good responders to tolbutamide Those in group two were termed moderate responders to tolbutamide – they were those patients who showed initial response but later on a partial relapse Group three contained the non-responders to tolbutamide who however never showed ketosis but who required

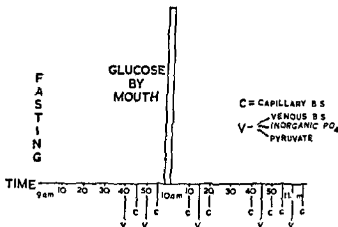


Fig 1 Glucose utilization test

small to moderate amounts of insulin Diabetics with a history of ketosis who required insulin constituted the fourth group A fifth group of non-diabetic subjects was included in the trial to serve as controls

The mean capillary venous difference prior to glucose administration was subtracted from the mean value of the differences estimated during 1 hour after glucose administration This final value was taken to be the measure of one of the effects of endogenous insulin Fig 2 shows the results of these tests in the five groups of subjects Of the diabetics the group responding to tolbutamide showed the best uptake after glucose but even so this was only approximately one-half that of the group of non-diabetic controls

There was no significant difference between moderate responders and non-ketotic non-responders to tolbutamide but the difference between non ketotic non-responders and good responders was significant The ketotic diabetics showed a negative uptake Among those non-diabetics initially chosen for the test were two with disseminated sclerosis two others with presumptive cortical or pituitary dysfunction These are shown in Fig 3 and are characterized by showing a negative uptake

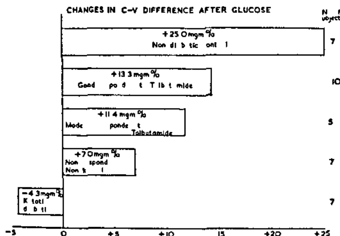


FIG 2

It might be considered that the rise of capillary blood sugar following the administration of glucose was a function of the metabolic abnormality Fig 4 shows a plot of the maximum capillary blood sugar following the administration of glucose plotted against the initial capillary blood-sugar concentration It can be seen that the maximum capillary blood-sugar level was correlated with the initial capillary blood sugar concentration and was independent of the type of diabetes under examination The figure also shows the narrowness of dispersion of the different groups along the regression line

Our results indicate that good responders to tolbutamide have a greater uptake of glucose than insulin-requiring diabetics and at the same time only approximately half the uptake of normal subjects This suggests to us that tolbutamide responders have more endogenous insulin than those who do not respond This has been confirmed by the plasma insulin assays of Vallance-Owen and others The tolbutamide-sensitive diabetics do not appear however to have so much insulin available as other non-diabetic subjects This may be due to less insulin being available in the pancreas to a

NEGATIVE C V CHANGES IN SELECTED NON DIABETIC SUBJECT

Case	Diagnosis	Initial capillary blood sug r mgm %	Initial C—V differenc mgm %	M n C—V diffe c fte glucose mgm %	Cha ge I C—V diff nce mgm %
1	Di semi at d Scl ro is	127	58	17	−41
2	Di semi t d Scl r is	72	8	−5	−13
3	D ml o t I b Sy d m	65	−4	−23	−17
4	Gross ob sity f ll l g M nl gitl	93	3	1	−2

Fig 3

slower release from the pancreas or to a greater rate of inactivation in the liver The finding of a negative glucose uptake both in ketotic diabetics and in certain normal subjects suffering from neurological disorders or ketotic dysfunction remains to be explained

It is of particular interest that six out of seven patients who had a history of ketosis had a negative glucose uptake We have no evidence whether this was due to the presence of some abnormal substance in the blood such as ketones or was due to simple passive pooling or stagnation of glucose on the venous side of the circula-

tion Observations on the occurrence of negative differences have been reported for many years At f thought to be due to technical errors but improvem chemical techniques have confirmed them but have our knowledge of their causation.

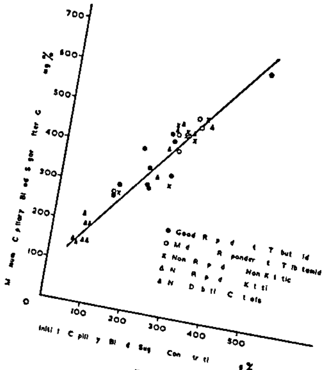


Fig 4

If the major function of the sulphonylureas is thought to be to stimulate or release endogenous insulin it would have been thought that its administration would alter the standard insulin sensitivity test only in those patients who still have a reserve of their own insulin. Fig 5 demonstrates the absence of the effect of carbutamide on insulin sensitivity tests of a young brittle diabetic with an 11 year history of the illness Fig 6 reviews the effect of carbutam

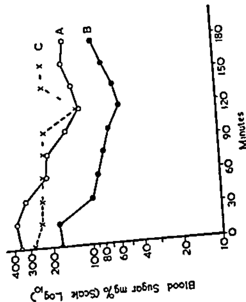

Mrs H age 56 Weight 45 kg Feb 1957 Plot
A IV Insulin O 3u/kg
B = IV Insulin + BZ55
C = BZ55 only
Blood Sugar mg% (Scale Log10)
400
300
200
100
80
60
40
20
10
0
30
60
90
120
150
180
Minutes
A
B
C

Fig 6

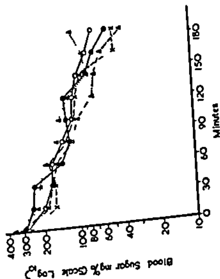

Miss L age 16 Weight 60 kg Jan Feb 1957 Plot
A IV Insulin O 3u/kg
B
C Insulin and D860 (1st week)
D (2nd week)
E (3rd week)
Blood Sugar mg% (Scale Log10)
400
300
200
100
80
60
40
20
10
0
30
60
90
120
150
180
Minutes

Fig 5

on the insulin-sensitivity test of a middle-aged diabetic Jamaican lady with an 8-year history of the illr omitted insulin at her own volition for 6 months prior A temporary lowering of the glucose plateau is show change in the sensitivity to insulin This patient eventua stabilization with insulin for permanent control Fig 7 apparent increase in sensitivity to insulin during tolb ministration in a thin young woman of 24 who had had h diagnosed only a few days prior to the test procedures and

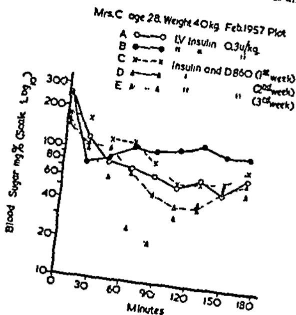


Fig 7

never before received insulin. The apparent increase in sensitivity to injected insulin over 3 weeks while on tolbutamide did not imply however that she might be controlled solely with the latter substance Such an attempt in fact failed and she required permanent insulin treatment for adequate stabilization

It is mere speculation to wonder whether the temporary increase in sensitivity was due to remnants of endogenous insulin being

stimulated by tolbutamide remnants however insufficient to ensure clinical control as shown by her eventual need of insulin

We are not able to separate this type of response from that of a true sparing of exogenous insulin as long as plasma insulin assays cannot differentiate between exogenous and endogenous insulin

SOME PERIPHERAL EFFECTS OF INSULIN AND OTHER HORMONES IN MAN

JEAN GINSBURG H-J B GALBRAITH and A PATON

Department of Medicine St Thomas s Hospital Medical School London

The fact that insulin has a marked action on extrahepatic tissues has been shown by many workers An increased uptake of glucose under the influence of insulin has been demonstrated in isolated tissues (Burn and Dale [8] Gemmill [17] Hechter *et al* [19] Hepburn and Latchford and Stadie *et al* [26]) and eviscerated preparations (Best *et al* [5] Burn and Dale [8] and Mann and Magath [-3]) The widening of the arterio-venous glucose difference which occurs after intravenous or intra arterial injections of insulin in animals and man (Andres and Zierler [1] Bell and Burns [4] Cori and Cori [9] and Frank *et al* [11 1- 13]) has also been taken as evidence of a peripheral effect

In the present investigation the effects of insulin hydrocortisone and glucagon have been measured in the forearm of healthy adults Peripheral responses to insulin were also studied in patients with diabetes acromegaly and other diseases associated with disturbances of carbohydrate metabolism and with an altered sensitivity to insulin

The forty-six subjects of the initial study were mainly medical students and colleagues nine convalescent patients with no evidence of metabolic or endocrine disturbance were also included in this group All subjects were tested at rest in the laboratory after a fast of 6 to 16 hours Simultaneous samples of arterial and venous blood were taken through needles maintained throughout the test in the brachial artery and antecubital vein of one arm and in the corresponding antecubital vein of the opposite arm Blood glucose was estimated by a modified Shaffer Hartmann method (Haslewood and Strookman[18]) In some tests hand or forearm blood flow were measured at half minute intervals using venous occlusion plethysmography (Barcroft and Swan[3]) It was thus possible to eval the influence of these hormones on glucose uptake in the

PERIPHERAL EFFECTS OF INTRA-ARTERIAL INSULIN (Figs 1 and 2)

After duplicate control samples had been taken 2 units of insulin diluted with isotonic saline to a volume of 2 ml were rapidly injected into the brachial artery Further samples were taken 5 15 30 45 and 60 minutes after the injection in all subjects and at 90 and 120 minutes in a few

The concentration of glucose in venous blood draining from the injected limb fell progressively after the injection of insulin to a mean value of only 35 mg per 100 ml at 30 minutes One hour after the insulin the mean level in this vein had risen slightly but was still 20-30 mg below control values When samples were taken 1½ to 2 hours after the intra-arterial injection of insulin glucose concentration in the ipsi-lateral draining vein was still well below control levels although glucose concentration had regained control values in arterial blood and blood from the contra-lateral vein

There was an initial fall in the glucose concentration of arterial blood and venous blood from the contra-lateral arm These changes were however much less than in the ipsi-lateral draining vein and control values were regained by the end of an hour at a time when the glucose content of venous blood from the injected limb was still below 60 mg per 100 ml

The selective and sustained fall in glucose concentration in the ipsi-lateral vein is due to the direct action of insulin and provides evidence that the hormone can be irreversibly fixed in healthy tissues *in vivo* as in isolated preparations (Stadie *et al*[26]) Fixation of insulin must have occurred with extreme rapidity for the injection was made in less than 2 seconds and the hormone could only have been in contact with the tissues of the forearm for a very few seconds

The effect of intra-arterial insulin was not confined to the tissues into which it was injected for the concentration of glucose also fell in arterial blood and in venous blood from the opposite arm though to a lesser extent These changes in glucose concentration presumably reflect the peripheral action of insulin after recirculation but may also result from the action of insulin in areas other than the extremities

There was no general or selective change in hand or forearm blood flow after intra-arterial injections of insulin Changes in arterio-venous glucose difference in the injected arm can therefore be used as a measure of the effect of intra-arterial insulin on glucose

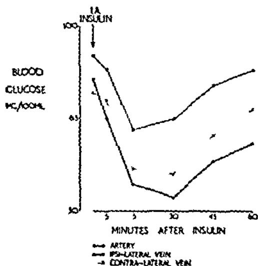


FIG 1 Effect of an intra-arterial injection of insulin (2 units) on arterial and venous glucose concentrat ns mean of fourteen experiments.

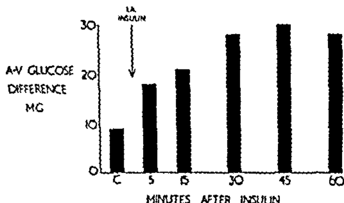


FIG. 2 Arterio-venous (A V) glucose differences in the injected arm after an intra-arterial injection of insulin (2 units) mean of twenty-two experiments.

uptake in that limb Initially (Fig 2) the arterio-venous glucose difference averaged 7 mg in both arms Five minutes after intra-arterial insulin the arterio-venous difference in the injected arm had increased to nearly 20 mg and remained elevated for a further hour at least

A selective increase in glucose uptake on the injected side could similarly be demonstrated when changes in the arterio-venous difference were expressed as a function of the arterial level of glucose using the formula A-V/A (Elrick *et al*[10])

PERIPHERAL EFFECTS OF HYDROCORTISONE (Figs 3 and 4)

The peripheral action of hydrocortisone was investigated in a similar manner *Hydrocortisone was given intra-arterially in doses* of 1-25 mg Samples were taken from the brachial artery and antecubital veins and blood flow was measured as before There was *no change in glucose concentrations or blood flow for 1 hour after* the injection It was therefore not possible to demonstrate any direct or immediate influence of hydrocortisone on glucose uptake in the forearm

Similarly (Fig 3) when 60-90 mg hydrocortisone were infused intravenously over 75 to 90 minutes there was no appreciable change in arterial glucose a slight increase in venous glucose was however recorded by the end of the hour when over 50 mg had been infused

The possibility that hydrocortisone might influence the action of insulin apart from any primary effect on the level of circulating glucose was also investigated

Hydrocortisone (0 6-1 mg /min) was given intravenously for 90 minutes Control samples were taken during the first 20 minutes of this infusion Two units of insulin were then injected into the brachial artery and samples taken as before for a further hour

The general pattern of response to intra-arterial insulin was much the same when hydrocortisone was infused (Fig 4) as when insulin was given alone (Fig 1) The mean changes in arterial glucose and arterio-venous glucose differences in the injected arm were not significantly different in the two groups Thus systemic administration of over 50 mg hydrocortisone did not affect the peripheral action of insulin or modify its fixation in forearm tissue

The prolonged administration of hydrocortisone or adrenocortical extracts undoubtedly cause a rise in blood sugar and altered

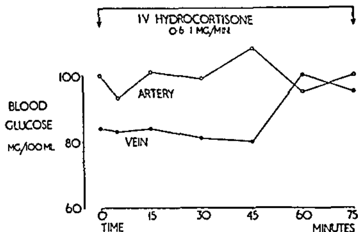


FIG 3 Blood glucose co centr n n dur g an intravenous infusion of hydrocortisone (0 6-1 mg /min) mean of four experiments

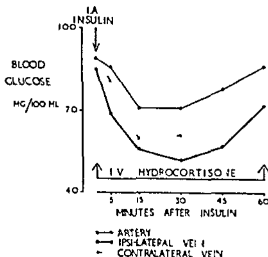


FIG 4 Effect of an intra-arterial injection of insulin (2 units) on arterial and venous b od glucose concentration during the i t venous infusion of hydrocortisone (0 6-1 mg /min.) me of twel e e periments

responses to insulin (de Bodo and Altszuler[6]) The precise mechanism is not clear but *there is much* evidence that the adrenal steroids influence carbohydrate metabolism more by an increase in hepatic gluconeogenesis than by a decrease in the peripheral uptake of glucose (Altszuler *et al* [2] Froesch *et al* [15 16]) The present findings would support this view The possibility that hydrocortisone may influence some phase of glucose utilization (Frawley [14] Henneman and Bunker [20] Hennes *et al* [21]) has not however been excluded by the present study

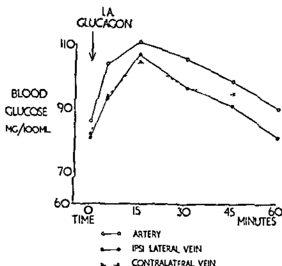


FIG 5 Effect of an intra-arterial injection of glucagon (0 02 mg) on arterial and venous blood glucose concentrations mean of fourteen experiments

PERIPHERAL EFFECTS OF GLUCAGON (Fig 5)

Glucagon* was given intra-arterially in a dose of 0 0_ mg *Blood* flow was measured and samples taken as before Glucose concentration rose simultaneously and equally *in the brachial artery and ante*cubital veins on either side Peripheral blood flow did not change after the injection of glucagon There was no selective rise or fall in the glucose level of venous blood draining the injected limb and no marked change in arterio-venous difference throughout It was therefore not possible to obtain evidence of a *direct peripheral* action of glucagon in man Since the general pattern of response

* The glucagon used in these experiments was supplied by *Eli Lilley & Co*

after arterial injection of the hormone was similar to that observed after intravenous administration any influence of glucagon alone on glucose uptake in the limbs would appear to be slight compared with its central effects

It has thus been possible to demonstrate in healthy adults a direct peripheral action of insulin and its fixation in forearm tissue In this respect insulin differs from the other hormones studied for there was no evidence that hydrocortisone or glucagon had any direct influence on the peripheral uptake of glucose

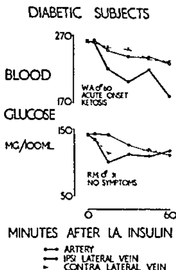


Fig 6 Effect of an intra-arterial injection of insulin (2 units) on arterial and venous blood glucose concentration in two patients with diabetes

The technique was then used to assess the peripheral response to insulin in patients with disturbances of carbohydrate metabolism and altered sensitivity to the hormone In view of the small numbers investigated at present and the difficulty of classifying patients with diabetes it is only possible to give selected examples of the response obtained

RESPONSES TO INTRA-ARTERIAL INSULIN IN DIABETIC SUBJECTS (Fig 6)

Nineteen patients between the ages of 26 and 74 were investigated shortly after diagnosis and before treatment with insulin

The peripheral action of insulin was modified in obese diabetic patients with an insidious onset of the disease and with absence of ketosis – the so-called insulin-resistant diabetic (R M Fig 6) In these subjects the concentration of glucose fell equally in the artery and antecubital veins on either side There was no selective fall in the glucose level of venous blood from the injected limb There was thus an impairment of the direct peripheral action of insulin and possibly failure of immediate local fixation

In patients with a recent acute onset of the disease who were subject to ketosis the fall in glucose concentration was greater in blood taken from the ipsi-lateral antecubital vein than in samples from the contra-lateral vein or artery The high initial level of blood sugar did not modify the direct peripheral action of insulin and local fixation of the hormone was apparent in these subjects (W A Fig 6)

In all diabetic subjects glucose concentration fell progressively in arterial and contra-lateral venous samples Fasting levels were not restored after an hour as in healthy subjects

It is tempting to speculate on the relation between these responses and the different clinical manifestations of the disease or the various inhibitory factors described in diabetic subjects (Bornstein [7] Vallance-Owen[27] [8]) The small size of the present series however precludes further discussion

RESPONSE TO INTRA-ARTERIAL INSULIN IN CUSHING S SYNDROME (Fig 7)

An altered response to intra-arterial insulin was observed in a patient with Cushing s syndrome secondary to adrenal hyperplasia The pattern of response was similar to that of obese diabetic subjects – an equal fall in arterial and venous glucose levels with no evidence of an immediate local action

Pituitary suppression was achieved by implanting radioactive yttrium Adrenal insufficiency and hypothyroidism developed after operation and maintenance prednisone and thyroid were given When the test was repeated 2 months after hypophysectomy the response to insulin was within normal limits There was a selective fall in venous glucose concentration in the injected limb and evidence of a local fixation of insulin

RESPONSE TO INTRA-ARTERIAL INSULIN IN ACROMEGALIC SUBJECTS (Fig 8)

The effect of intra-arterial insulin was studied in six patients with acromegaly the disease was active in three cases and quiescent in the remaining three

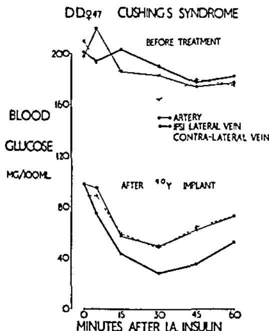


Fig 7 Effect of an intra-arterial inject on of insulin (2 unit) on arterial and venous bl od glucose concentr tions in p tient w th Cushing s syndrome befo d after hypophy sect my

Fasting blood sugar was only raised in one patient but the peripheral action of insulin differed from normal in all six patients Glucose concentration in the draining ipsi lateral vein fell initially in five patients but control values were rapidly regained The fall in the arterial and contra-lateral venous levels of glucose was slight by comparison with normal Indeed in one patient there was no fall in glucose concentration in either vein or artery

The altered response in patients with acromegaly was thus not

dependent on activity of the disease or on a raised level of circulating glucose Furthermore the abnormality was distinct from that observed in obese diabetic patients

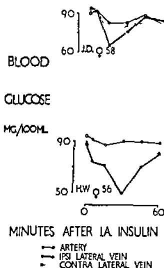


FIG 8 Effect of an intra arterial injection of insulin (2 units) on arterial and venous blood glucose concentrations in two patients with acromegaly

SUMMARY

1 The direct peripheral action of insulin and its fixation in healthy tissues has been demonstrated in fasting adults the uptake of glucose in the forearm was increased for at least 2 hours after an intra-arterial injection of insulin This effect was not modified by the simultaneous intravenous infusion of hydrocortisone

2 Hydrocortisone and glucagon had no direct influence on the peripheral uptake of glucose

3 Peripheral responses to insulin were modified in obese diabetic subjects a similar response was observed in a patient with Cushing s syndrome

4 A different abnormality in the peripheral response to insulin was observed in patients with acromegaly

5 Further studies are required to determine the relation between these altered responses to insulin and the presence or absence of specific factors in conditions of endocrine imbalance

REFERENCES

1 ANDRES R. & ZIERLER K. L. (1956) *Amer J Ph ys ol* 187 583
2 ALTSZULER, N STEELE, R WALL J S & DE BODO R. C (1958) *Amer J Phy iol* 192 219
3 BARCROFT H. & SWAN H. J C (1953) *Sympatl t c C tr l f H man Blood l essels* London.
4 BELL D M & BUR S T (1952) *J Cl I est* 31 717
5 BEST C. H. DALE H H HOET J P & MARKS H P (19_6) *Proc Roy Soc B* 100 55
6 DE BODO R C & ALTSZULER N (1958) *Phys l Rev* 38 389
7 BORNSTEIN J & TREWHELLA P (1950) *A tr l J Exper B ol* 28 573
8 BURN J H & DALE, H H (1924) *J Physi l* 59 164
9 CORI C F & CORI G T (1925) *Amer J Phys ol* 71 688
10 ELRICK, H HEAD C J & WITTEN T (1955) *J Cl I e t* 34 1830
11 FRANK E NOTHMANN M & WAGNER A (1924) *Kl W hsch* 3 581
12 FRANK E NOTHMANN M & WAGNER A (1924b) *Kl W hschr* 3 1404
13 FRANK E NOTHMANN M & WAGNER A (1925) *Ar h f Exper Path u Pharmak l* 110 225
14 FRAWLEY T F (1955) *A N Y Acad S* 61 464
15 FROESCH E R. ASHMORE, J & RENOLD A E. (1958) *Endocrinology* 62 614
16 FROESCH E. R WINEGRAD A I RENOLD A E. & THORN G W (1958) *J Cl I est* 37 524
17 GEMMILL, C L (1940) *B ll Johns H pk H p* 66 232
18 HASLEWOOD G A D & STROOKMAN T A (1939) *B o hem J* 33 920
19 HECHTER O LEVINE, R. & SOSKIN S (1941) *Proc Soc exper B l & M d* 46 390
20 HENNEMAN D H & BUNKER J P (1957) *Amer J Med* 23 34
21 HENNES A R WAJCHENBERG B L FAJANS S S & CONN J W (1957) *M t bolism* 6 339
22 HEPBURN J & LATCHFORD J K (1922) *Amer J Phys ol* 62 177
23 MANN F C & MAGATH T B (1923) *Amer J Ph y iol* 65 403
24 STADIE, W C (1951) *A N Y A ad Sc* 54 671
25 STADIE, W C HAUGAARD N & MARSH J B (1952) *J b ol Chem* 198 785
26 STADIE, W C. HAUGAARD N HILLS A G & MARSH J B (1949) *Amer J Med Sci* 218 265
27 VALLANCE-OWEN J HURLOCK, B & PLEASE, N W (1955) *Lancet* ii 583
28 VALLANCE-OWEN J DENNES E. & CAMPBELL, P N (1958) *Lancet* ii 336

SALICYLATE IN DIABETES MELLITUS

J Reid and T D Lightbody

Western Infirmary Glasgow

Interest in the action of salicylate in diabetes mellitus has been revived by the observation that aspirin given in the treatment of acute rheumatism also controlled the glycosuria and hyperglycaemia of a young diabetic This action was confirmed in another group of diabetics and in addition to controlling glycosuria and hyperglycaemia aspirin was found to be capable of abolishing moderately severe ketosis[4]

Disadvantages of the therapy are early development of nausea and vomiting in a few patients and milder but more frequent undesirable effects such as tinnitus and deafness though the latter tend to disappear with time Nausea and vomiting are due to unsuspected overdosage with salicylate and are associated with very high serum salicylate levels They are serious because they give rise to a starvation ketosis which is difficult to distinguish from diabetic ketosis and this confusion probably accounts for the abandonment of salicylate in diabetes long before the introduction of insulin Fortunately nausea and vomiting need not arise if salicylate dosage is controlled by serum salicylate estimations in the early days of treatment so that none of the disadvantages really presents an insuperable problem to its use in diabetes Indeed the fact that salicylate s long and continuous administration is not accompanied by risk of serious toxic effects such as agranulocytosis is in its favour for while this risk may be negligible in short term treatment it is not in a treatment which lasts for life The practical value of aspirin in diabetes however cannot be decided until its potency has been compared with insulin the most effective anti-diabetic agent known

We have investigated the insulin equivalence of aspirin in a group of fourteen patients whose insulin requirements ranged from 14 to 112 units each day The patients were referred to us from the hospital diabetic clinic Some were well controlled with insulin but a good proportion of difficult cases who were not well controlled included in the series The complete practical range of

requirements has been covered and it may fairly be claimed that the group represents a cross-section of diabetics who require insulin

Clinical particulars are shown in Fig 1 Ages ranged from 33 to 74 years Nine were females five were males The duration of the disease varied from 1 to 24 years the carbohydrate intake from 104 to 295 g daily and total calories from 1300 to 2700 The diets which the patients were accustomed to take as out-patients were continued throughout their whole stay in hospital that is for the

CLINICAL PA TICULA S

Pa i t	Age	S	Dur D be es	OUT IE T Do I su R q nt unit	D ET Gm. Carb./day	Total Cals./day
K.L.	47	F	4	1 unit	104	300
S.M'R.	50	F	4	20 units	120	1400
A.C.	8	M		20 units	97	2100
S.M.	45	M	4	22 units	209	2 00
E.J.	55	M	1	24 unit	50	1900
S.W.	65	F	13	28 unit	145	700
J.C.	46	F	6	36 units	150	1700
S.H.	74	F	7	38	104	1400
C.M.P.	47	F	6	40	198	2000
H.M.M.	58	F	7	56 units	130	1500
D.L.	48	M	3	56 units	224	2300
M.C.	74	F	12	60	104	1300
R.C.	33	M	20	76 units	295	2700
C.M.R.	51	F	8	112 units	207	2000

Fig 1

duration of the investigation except in one patient in whom a slight alteration was made during the insulin period The only material change in insulin administration was to replace protamine zinc insulin with lente insulin in three patients so that all received the same type of insulin Therapy was controlled by fasting blood sugar estimations and tests for glycosuria throughout the day After an initial control period of at least 10 days during which the pat received their appropriate doses of insulin aspirin therapy wa started and insulin therapy continued Aspirin was given in to attain maximum therapeutic serum salicyla

mg /100 ml Fasting blood sugar and urinary sugar estimat continued and insulin dosage was progressively reduced if wa by the biochemical findings The amount of insulin which be replaced by maximum therapeutic doses of aspirin wa determined for each patient

Results are presented in Fig - where it will be noticed that

NSU IN EQU LE CE O SP R N

		D T S C M		DURING S R N 2 3		
ENT	D ET CHO Gm/Day	SUL REQU EME U L /Day	S G BLOOD SUG M 100ml	FASTING BLOOD SUGAR Mg/ 00ml	NS EQU EMENT U /Day	D ERENCE NS L N REQUIREME
S M R	10	2	00	00	0	2
R L	08	4	10	00	0	
C	17	10	2 2	60	0	20
J J	10	24	184	81	0	2
S W	85	28	219	90	0	28
J C	10	36	10	7 1	0	26
S N	0	36	85	50	0	36
M C	04	8	48	85	0	8
R M	204	22	8 272	25 100	8	0
C M P	98	32	96 286	73 226	8	6
M M M	20	8	53 760	9 286	28	4
B L	186	52	51 171	40 190	40	2
R C	279	76	73 283	7 285	56	20
C M R	207	3	289	19	40	32

D N W T Gm C D 6 1 M

Fig 3

possible to reduce insulin requirement of every patient after - to 3 weeks treatment with aspirin

Eight patients with an initial requirement of 1- to 48 units of lente insulin were able to do without insulin altogether while aspirin was being given Their fasting blood sugars at the end of the insulin and the aspirin periods were quite stable

The next five patients had a modest reduction in insulin requirement of 10 to -0 units of lente insulin but the lability of the fasting

Fig 5 presents results in another fairly stable diabetic requiring the largest dose of lente insulin – 112 units per day Insulin requirement in this case was reduced to 40 units per day and this probably is an understatement because fasting blood sugar and glycosuria were lower during aspirin therapy than they were in the control period with insulin alone The insulin equivalence was therefore more than 72 units per day

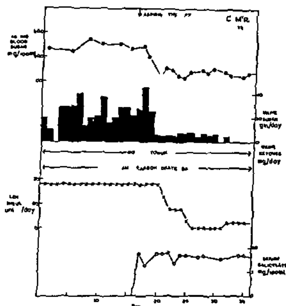


Fig 5 Findings in patient who was stabilized rather than controlled w th 112 units of I Z S The main effects of aspirin were a fall in fasti ig blood sugar to normal and diminution in *glycosuria, accompanied by a reduction in insulin requirement to 40 units daily*

Finally Fig 6 illustrates what happens when the aspirin course precedes insulin administration The high fasting blood sugar is quickly brought under control and there is negligible glycosuria during the period of aspirin therapy After stopping aspirin the blood sugar takes just about as long to reach pre-aspirin level as it took to come down to normal Thirty-six units of lente insulin per day were required to maintain the fasting blood sugar at a slightly higher control level

In summary aspirin in full therapeutic doses is not so potent as insulin The greatest amount of lente insulin which could be replaced by aspirin was more than 7_ units per day and this in the patient with the greatest insulin requirement The maximum insulin requirement which could be completely replaced by aspirin was 48 units per day but responses like these were only obtained in

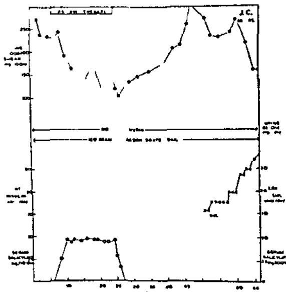


FIG 6 Course of even s when p in therapy precedes insul i. Fasting blood sugar return to norm l d ring asp n admi ist t on only to revert to diabeti lev ls after stopping the drug Thirty-six un ts of nsul n were required to bring the f sting blood sugar level back to a slightly h gher control lev l than with a pirin.

patients who were well controlled with lente insulin alone Patients who were difficult to control with lente insulin were equally difficult to control with aspirin Another point worth mentioning is that the increasing response to aspirin as insulin requirement becomes greater suggests that the frequency of hypoglycaemic attacks in patients requiring large daily quantities of insulin may be reduced by combined aspirin/insulin therapy and the fact that hypoglycaemia is not a feature of aspirin therapy may have a bearing on this

These results place salicylate in the class of promising compounds for oral treatment of diabetes and justify carrying on investigations firstly to try to overcome the disadvantages of salicylate therapy already referred to and secondly to find out how it acts The first of these investigations is under way The second to find out how salicylate acts in diabetes will now briefly be considered

In general there are two approaches to work of this kind – the biochemist s and the physician s The biochemist tends to start with a simple biological preparation and work up to the whole organism The research physician works in the opposite direction from the diseased patient to the normal and then to a simpler biological system So far as diabetes is concerned the two approaches are mutually advantageous and this is exemplified by the recent findings of Manchester *et al* [9] who noted that salicylate like insulin increased glucose uptake by isolated rat diaphragm Unlike insulin however salicylate increases the potassium loss and diminishes the amino-acid incorporation in protein and it is not without interest that these biochemical observations on isolated muscle agree with the changes from salicylate we have observed in rheumatic patients [3]

For our part it would perhaps not be so profitable to concentrate exclusively in looking for an explanation of salicylate s action in diabetes Our interest in diabetes arose by chance while investigating the therapeutic action of the drug in rheumatic fever and in probing other possible therapeutic applications for it This work was undertaken mainly to find out more about the nature of the disease processes It would not however be appropriate now to do more than direct your attention to the amazing therapeutic versatility of this comparatively simple substance and then suggest in general terms how we might reduce it to understandable proportions

Fig 7 lists three disease states which are influenced by salicylate They are rheumatic fever diabetes mellitus and myxoedema No apparent connection between the diseases is known All three are also controlled by naturally produced hormones but each hormone benefits only one disease while salicylate affects them all though its range of action and potency varies It is the drug of choice in rheumatic fever It is not so potent as insulin in diabetes and it only affects some but not all of the abnormalities in myxoedema but its cholesterol-[1] ...ng effect in this disease is most pronounced and this prc... just as effective as thyroid hormones [1] More- ... it has ... noted that salicylate lowers the cholesterol

level in other hypercholesterolaemic states including diabetes so that it would be well to keep this effect in mind when considering the problem of vascular degeneration — an important complication in diabetes

DISEASE	THERAPEUTIC AGENT
RHEUMATIC FEVER	ASPIRIN CORTISONE
DIABETES MELLITUS	ASPIRIN INSULIN
MYXOEDEMA LOWERING OF CHOLESTEROL	ASPIRIN THYROXINE

FIG 7

I am convinced that another approach to the problem of how salicylate acts in diabetes as well as in the other diseases is to continue the search for the fundamental biological action of the drug and to trace the whole series of effects which stem from it for it has been the more or less blind utilization of these individual effects for therapeutic purposes which in our present ignorance of their interrelations makes us wonder at and perhaps even doubt its remarkable therapeutic properties

REFERENCES

1 ALEXANDER W D & JOHNSON K. W M (1956) *Clinical Science* 15 593
2 MANCHESTER, K L RANDLE, P J & HOWARD SMITH, G (1958) *Brit med J* I, 1028
3 REID J WATSON R. D & SPROULL D H (1950) *Quart J Med* N S 19 1
4 REID J MACDOUGALL A I & ANDREWS, M M (1957) *Brit med J* II, 1071

These results place salicylate in the class of promising compounds for oral treatment of diabetes and justify carrying on investigations firstly to try to overcome the disadvantages of salicylate therapy already referred to and secondly to find out how it acts The first of these investigations is under way The second to find out how salicylate acts in diabetes will now briefly be considered

In general there are two approaches to work of this kind – the biochemist s and the physician s The biochemist tends to start with a simple biological preparation and work up to the whole organism The research physician works in the opposite direction from the diseased patient to the normal and then to a simpler biological system So far as diabetes is concerned the two approaches are mutually advantageous and this is exemplified by the recent findings of Manchester *et al* [2] who noted that salicylate like insulin increased glucose uptake by isolated rat diaphragm Unlike insulin however salicylate increases the potassium loss and diminishes the amino-acid incorporation in protein and it is not without interest that these biochemical observations on isolated muscle agree with the changes from salicylate we have observed in rheumatic patients [3]

For our part it would perhaps not be so profitable to concentrate exclusively in looking for an explanation of salicylate s action in diabetes Our interest in diabetes arose by chance while investigating the therapeutic action of the drug in rheumatic fever and in probing other possible therapeutic applications for it This work was undertaken mainly to find out more about the nature of the disease processes It would not however be appropriate now to do more than direct your attention to the amazing therapeutic versatility of this comparatively simple substance and then suggest in general terms how we might reduce it to understandable proportions

Fig 7 lists three disease states which are influenced by salicylate They are rheumatic fever diabetes mellitus and myxoedema No apparent connection between the diseases is known All three are also controlled by naturally produced hormones but each hormone benefits only one disease while salicylate affects them all though its range of action and potency varies It is the drug of choice in rheumatic fever It is not so potent as insulin in diabetes and it only affects some but not all of the abnormalities in myxoedema but its cholesterol-lowering effect in this disease is most pronounced and in this property it is just as effective as thyroid hormones [1] Moreover it has recently been noted that salicylate lowers the cholesterol

level in other hypercholesterolaemic states including diabetes so that it would be well to keep this effect in mind when considering the problem of vascular degeneration – an important complication in diabetes

DISEASE	THERAPEUTIC AGENT
RHEUMATIC FEVER	ASPIRIN CORTISONE
DIABETES MELLITUS	ASPIRIN INSULIN
MYXOEDEMA LOWERING OF CHOLESTEROL	ASPIRIN THYROXINE

FIG 7

I am convinced that another approach to the problem of how salicylate acts in diabetes as well as in the other diseases is to continue the search for the fundamental biological action of the drug and to trace the whole series of effects which stem from it for it has been the more or less blind utilization of these individual effects for therapeutic purposes which in our present ignorance of their interrelations makes us wonder at and perhaps even doubt its remarkable therapeutic properties

REFERENCES

1 ALEXANDER W D & JOHNSON K W M. (1956) *Cl S* 15 593
2 MANCHESTER, K L RANDLE, P J & HOWARD SMITH G (1958) *B t m d J* i 1028
3 REID J WATSON R. D & SPROULL D H (1950) *Q rt J Med* N S 19 1
4 REID J MACDOUGALL A I & ANDREWS M M (1957) *B t m d J* ii 1071

THIOL SUBSTANCES AND DIABETES MELLITUS

W J H Butterfield

Guy s Hospital London

Sulphydryl substances are precursors in the synthesis of insulin and are also involved at various stages in intracellular carbohydrate metabolism Lazarow[13] has reviewed the former question and discussed the steps whereby the β cells elaborate insulin from thio-amino acids such as cystine which constitutes over 10 per cent of the insulin molecule and discussed possible ways whereby diabetogenic substances for example alloxan interfere with these processes I do not intend to go further into this here but shall consider in more detail the other aspect that is intracellular reactions which depend upon thiol-containing substances

These reactions include the conversion of glyceraldehyde-3-phosphate into 1 3 diphosphoglyceric acid by triosephosphate dehydrogenase which enzyme includes glutathione the cleavage of one carbon atom from pyruvate which involves a dithiol α-lipoic acid acetylation which involves Co-enzyme A and in the Kreb s cycle first the conversion of α-keto-glutarate to succinate also involving α lipoic acid and Co-enzyme A and second the conversion of succinate to fumarate which involves succinic dehydrogenase In addition Long *et al*[14] have published evidence that the phosphoglucomutase system may be thiol dependent

My purpose is to review some pieces of evidence accumulated since 1950 suggesting that diabetes may be associated with an abnormality of thiol metabolism affecting some of these steps The two points in carbohydrate metabolism on which I shall be focusing attention are first the action of insulin in facilitating the entry of glucose into cells and second the accumulation of pyruvate in the cells and its diffusion into the blood if it is not properly handled by being incorporated by intermediary metabolic processes and oxidized or stored as fat

I DIABETES AFTER INJURY

The first piece of evidence is based on abnormal carbohydrate metabolism after injury already alluded to briefly in the literature [3 8]

This study was concerned with burns a form of injury which permits the investigator roughly to assess the severity from the percentage of the body burned

Over a period of 18 months the burn service of the Medical College of *Virginia in Richmond* admitted 140 cases with burns of second or third degree of over 10 per cent body surface requiring resuscitation and skin grafting These cases include patients of ages 2-81 years male and female white and coloured All were resuscitated by a standard treatment and as soon as the initial phase of circulatory collapse was over (about 2 days) the patients received a high-protein high-caloric diet to offset the weight-loss so frequently observed after burns

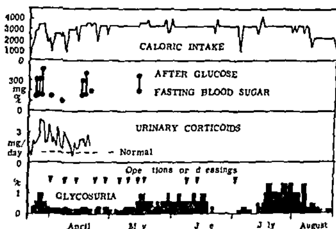


FIG 1 An example of glycosuria following thermal injury

The present study began when I found by chance that one of these patients previously aglycosuric was passing sugar in her urine *several days after being admitted to hospital* Thereafter arrangements were made to test *24-hour urine collections for glycosuria* on all burns At the end of 18 months of the total of 140 cases admitted 10 per cent had developed glycosuria some days *after* their injury Since then several other cases have been *observed in this country* and colleagues in London Birmingham Edinburgh and various centres in the U S have also noted a similar condition

Fig 1 gives relevant information about an example case The

patient suffered very deep flame burns of 19 per cent of his body surface was resuscitated in the usual way and feeding was started by the dietary service The calories fed are indicated The intensity of the glycosuria *which started a feu days after injury* is also indicated It will be observed that glycosuria persisted for about 4 months It was aggravated by surgical procedures dressings or operations Some blood sugar estimations are recorded on this chart to indicate that the glycosuria was not renal in origin Also included are estimates of the urinary formaldehydrogenic corticoid excretion[7] which was elevated above normal levels I shall return to this point presently

Table I below summarizes the clinical findings in the cases of glycosuria following burns

TABLE I

SUMMARY OF 140 BURN CASES ADMITTED DURING 18 MONTHS
FOURTEEN DEVELOPED THE DIABETIC STATE
(ght m l six f males)

	R ge	*Mean*
Age	8 76	40
P cent burn	11 59	34
D y of o et of glyco uria	3–9	6
Blood sug		
fasting	100–279	151
1 hour	199–450	3 5
A t nuri	No e	
D t n of di betic state	Sh rte t in children Longest in the elde ly	

Of the possible causes for this diabetic state three came to mind early in the investigations Since the first few cases had been resuscitated with a polysaccharide dextran one was concerned lest the diabetes be a toxic result of this substance However it was presently traced in cases who did not receive any dextran A second possibility was that the diet might be the cause of the diabetes To ensure that the diet was not harmful *per se* eight volunteers were put to bed bandaged with burn dressings and given the same treatment for 10 days None showed glycosuria all gained weight Clearly the high diets* alone were not the cause of the glycosuria

* Obvio ly the d mands by the surgeons f r f feed g were completely contrary to the st p whi h the treatment of hypergly mia required, but since these patients h d to be k pt in good nutriti al cond tion f a long series of grafting operati ns, th surgical requirement tended to take p cedence see [8] p 613

The other possibility was that these patients had hyperadrenocorticism It was recognized that severe thermal injury is a potent stimulus to the adrenocortical axis [2] so adrenocortical function was assessed in nineteen burns and in eight volunteers Table II summarizes the results for the first 5 days after injury in (i) the nine burns who subsequently developed glycosuria (ii) the ten burns who did not develop diabetes and (iii) the eight control subjects

The results furnish good evidence that the group who developed diabetes temporarily had hypercorticism Indeed in this respect one might regard them as similar to pregnant women in whom Hoet[10] and Jackson and Wolff[11] have observed abnormal carbohydrate metabolism

TABLE II

STUDIES OF ADRENOCORTICAL FUNCTION DURING FIRST 5 DAYS AFTER INJURY

	Nine burns with diabetes	*Ten burns without diabetes*	*Controls (eight)*
Mean age	37	22	34
Mean per cent burn	36	14	—
Mean corticoids mg/day	2 2	1 4	0 7
Mean eosinophils cu mm	6	30	105

Carbohydrate metabolism within 3 weeks of injury Turning to the special investigations of carbohydrate metabolism glucose (50 g glucose by mouth) and glucose-insulin (50 g glucose by mouth 5 units insulin i v) tests were performed If tested within 3 weeks of injury these cases showed diminished glucose tolerance and absence of response to insulin in the glucose-insulin test (Fig 2) Similar results are found in Cushing s disease or during ACTH and cortisone treatment

The question arose what was the cause of the insulin resistance? Possibilities included insulin antagonists or some failure in intracellular carbohydrate metabolism It was decided to test these two general possibilities by measuring the blood pyruvate levels in conjunction with the glucose and glucose-insulin tests The results (Fig 3) showed (i) abnormally high fasting levels and (ii) abnormally high rises during the glucose test The pyruvate levels and rises

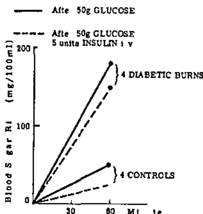


FIG 2 Mean rise of blood-sugar l v l during glu and gl cose-insulin in burns with hype gly mi and in c ntrol

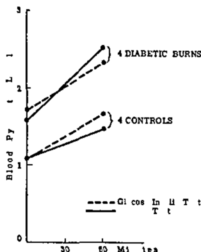


FIG 3 M an pyru ate l ls (mg/100 g blood) in gluco and glucose-insulin tests in burns w th hyperglycaemia and in c trol

were much greater than observed in non-diabetic burns even of equal severity or extent or in controls or in insulin-dependent diabetes[3]

It was therefore difficult to reconcile these findings with any explanation for insulin resistance based on insulin antagonists during the glucose and glucose-insulin tests glucose must have penetrated the cells and been broken down to pyruvate which then diffused back into the blood stream The accumulation of the pyruvate observed was accepted as evidence that there was some alteration in intracellular carbohydrate metabolism *in some tissue or tissues*

The notion that the accumulations of glucose and pyruvate in the blood could be due to the excessive diet had already been dispelled but the contrast between the controls who tended to gain weight on high calorific diets and the diabetic burns who tended to lose weight and go into negative nitrogen balance offered a clue The diabetic burns exhibited a metabolic block presumably at or below the level of pyruvate oxidation which interfered with the usual relationship between carbohydrate consumption on the one hand and fat deposition and protein sparing on the other

About that time Conn and his colleagues reported that intravenous glutathione had lowered the blood sugar of a female volunteer made diabetic by ACTH injections[6] The pyruvate oxidation system being dependent upon two thiol substances lipoic acid and Co-enzyme A it was decided to explore the effects of BAL on the glucose tolerance in (i) the diabetic burned patients (ii) patients who were burned but not diabetic and (iii) in non-diabetic controls 150 mg of BAL were given i m shortly before the start of the usual glucose tolerance test In the latter two groups *BAL was without effect* but in some of the diabetic burns the thiol appeared to improve glucose tolerance (Fig 4) Furthermore the rise in blood pyruvate was smaller in the BAL-glucose test than when glucose was given alone

It should be stressed that these results obtained *only in* patients who were tested within *3 weeks of their injury* after that time the diabetic burns became more insulin-sensitive less responsive to BAL alone but did respond when treated with both insulin and *BAL*[3]

The tentative conclusion was that an abnormality of SH metabolism *operating at the level of pyruvate* oxidation and most pronounced soon after the injury would explain the findings It seemed unlikely that the patients could be suffering from a deficiency of

the monothiol Co-enzyme A since they were able to break down and presumably utilize the body depot fat On the other hand the results with the dithiol BAL suggested that the biochemical lesion was associated with the inability of pyruvate to enter intermediary metabolism a step which involves the natural dithiol α lipoic acid

One other point remained for examination Was a similar biochemical lesion detectable in non diabetic burns made glycosuric by ACTH? Hyperpyruvataemia has been reported after ACTH and cortisone by several investigators [9 L] In the course of a skin-grafting trial ACTH was given to a burn Hyperpyruvataemia and sensitivity to BAL were found [4]

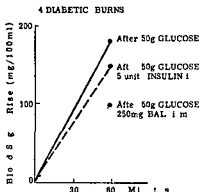


FIG 4 M an rises of blood-sugar levels in gl o e glucose-insulin d gl co e BAL toler nce tests

The order of the day at this symposium is speculation Setting aside the fact that these investigations were carried out in burned patients where many pathological factors operate – infection tissue necrosis and so on – and assuming that the action of BAL is on SH metabolism and not due to any kelating effect [16] it is tempting to suggest that stress situations may be associated with a change in SH metabolism[1] If this affected all tissues for a few weeks it would explain the insulin resistance and the hypoglycaemic effect of BAL Later on this metabolic block might be expected to leave its mark on the function of the β-cells themselves interfering with their utilization of glucose and therefore presumably with their production of insulin This would explain the subsequent insulin sensitivity

Various aspects of the foregoing investigation have been studied in established medical as opposed to traumatic diabetes as follows

2 EFFECT OF THIOL SUBSTANCES IN INSULIN-RESISTANT DIABETES

One of the features of the diabetes described above after burns was insensitivity to insulin in the glucose-insulin test Two diabetic cases showing marked insulin resistance have therefore been studied for the effects of thiol substances *

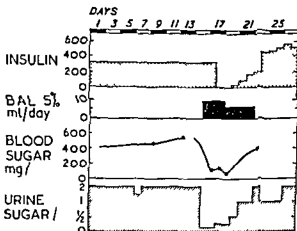


FIG 5 Effect of BAL on blood sugar and glycosuria in a 37 year-old diabetic

The first was a 37-year-old plump coloured woman a severe diabetic of many years standing who had been treated with gradually increasing doses of insulin without satisfactory control She was suffering from diabetic neuritis and Kimmelstiel-Wilson s syndrome She showed intense glycosuria but no acetonuria her blood sugar rising up to 540 mg per cent (Fig 5) After a suitable control period on a fixed diet and 320 units of insulin/day BAL (150 mg in oil i m three times a day) was started in addition to the insulin Next day her urine showed only a trace of glucose and her fasting blood sugar was 118 mg per cent The following day insulin was withheld but BAL continued There was a trace of sugar in the urine the fasting blood sugar was 140 mg per cent rising to

* These are cases 1 and 2 in [4] Figs 5 and 6 from that publication.

190 mg per cent 1 hour after 50 g glucose by mouth indicating greatly enhanced glucose tolerance

On the fourth day the fasting blood sugar was 81 mg per cent and there was very little glycosuria

When BAL was stopped intense glycosuria reappeared immediately and the blood sugar rose to 400 mg per cent despite re-instituting high doses of insulin

The second case was similar A 36-year-old white woman a severe obese diabetic of 15 years standing with diabetic neuritis and Kimmelstiel-Wilson s syndrome was uncontrolled by large doses of insulin varying up to 2000 units/day she showed no acetonuria She was admitted to hospital for study Examination of her urine for α keto acids by Dr Brian McArdle showed high values of both pyruvate and α ketoglutarate (see Table III) which may be inter-

TABLE III

MEAN URINARY EXCRETIONS OF PYRUVATE AND α KETOGLUTARATE

	P t t	C trols	P
Pyruv t mg	23	6 9	< 001
ketogl t te mg	63 5	35 4	< 001

preted as evidence for impaired metabolism of these intermediary compounds both of which require lipoic acid for their metabolism

After a control period on a fixed diet of 1600 calories and ‿80 units of insulin/day BAL (‿00 mg t d s) was given for 3 days (see Fig 6) A prompt reduction in blood sugar and urine sugar occurred When the BAL was withdrawn the blood and urine sugars rose to previous values After 5 days BAL was again administered (200 mg four times/day) There was again a fall in blood sugar and glycosuria

This case was studied intensively over a period of 3 months The response to various doses of BAL for 3 or more days was studied the patient being maintained on diets of 1600 and 800 calories a day The mean daily glycosuria on the various experimental conditions different diet and altered doses of BAL are shown in Fig 7 It will be seen that the effect of BAL was progressively greater as the dose was increased

Various aspects of the foregoing investigation have been studied in established medical as opposed to traumatic diabetes as follows

2 EFFECT OF THIOL SUBSTANCES IN INSULIN-RESISTANT DIABETES

One of the features of the diabetes described above after burns was insensitivity to insulin in the glucose-insulin test Two diabetic cases showing marked insulin resistance have therefore been studied for the effects of thiol substances *

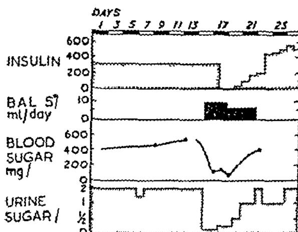


Fig 5 Effect of BAL on blood sugar and glycosuria in a 37 year-old

The first was a 37-year-old plump coloured woman diabetic of many years standing who had been ᵗʳ gradually increasing doses of insulin without satisfacto She was suffering from diabetic neuritis and Kimmel syndrome She showed intense glycosuria but no ac blood *sugar rising up to 540 mg per cent* (Fig 5) A control period on a fixed diet and 3_0 units of insuli (150 mg in oil 1 m three times a day) was started 1 the insulin Next day her urine showed only a trace c her fasting blood sugar was 118 mg per cent The ^ insulin was withheld but BAL continued There was a in the urine the fasting blood sugar was 140 mg

* These are cases 1 and 2 in * Figs 5 and 6 from that pub

190 mg per cent 1 hour after 50 g glucose by mout greatly enhanced glucose tolerance

On the fourth day the fasting blood sugar was 81 and there was very little glycosuria

When BAL was stopped intense glycosuria r mediately and the blood sugar rose to 400 mg per c re-instituting high doses of insulin

The second case was similar A 36-year-old white severe obese diabetic of 15 years standing with diabetic r Kimmelstiel-Wilson s syndrome was uncontrolled by la of insulin varying up to ,000 units/day she showed no ac She was admitted to hospital for study Examination of l for α-keto acids by Dr Brian McArdle showed high values pyruvate and α-ketoglutarate (see Table III) which may be

TABLE III

MEAN URINARY EXCRETIONS OF PYRUVATE AND α KETOGLUTARATE

	P t nt	C nt l	P
Pyruvate n g	23	69	< 001
α-k toglut at mg	63 5	35 4	< 001

preted as evidence for impaired metabolism of these interm compounds both of which require lipoic acid for their metabolism

After a control period on a fixed diet of 1600 calories and ,80 of insulin/day BAL (,00 mg t d s) was given for 3 days (see Fig 6) A prompt reduction in blood sugar and urine sugar occurred When the BAL was withdrawn the blood and urine sugars rose to previous values After 5 days BAL was again administered (200 mg four times/day) There was again a fall in blood sugar and glycosuria

This case was studied intensively over a period of 3 months The response to various doses of BAL for 3 or more days was studied the patient being maintained on diets of 1600 and 800 calories a day The mean daily glycosuria on the various experimental conditions different diet and altered doses of BAL are shown in Fig 7 It will be seen that the effect of BAL was progressively greater as the dose was increased

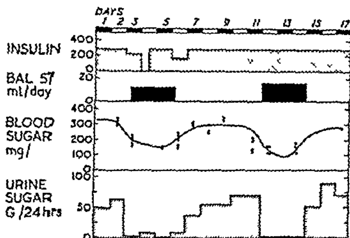
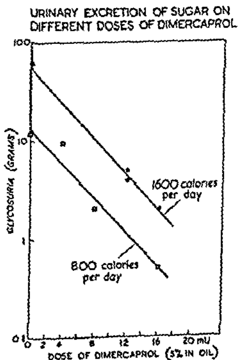


Fig 6 Effect of BAL on blood sugar and glycosuria in a 36-year-old diab-



Effect of BAL on 24 hour glycosuria (i) diet 1600 cal./

About 12 months later the opportunity arose to study the effect of another SH compound in this patient. A small supply of mercaptoethylamine salicylate was made available to us.* Ignorant of the sort of dose regime to use we started with 100 mg † capsules four times a day given when the patient was out of control on 1000 units insulin/day (Fig 8). After a delay of 3 days during which no response was detected the patient unexpectedly became normoglycaemic and the thiol was stopped. Blood-sugar levels were in

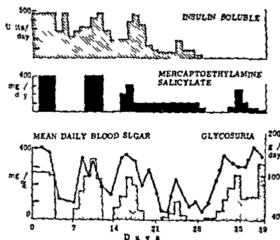


Fig 8 Effects of mercaptoethylamine salicylate in an insulin-resistant diabetic

the normal range for 2 days after which the blood and urine sugar values rose. Mercaptoethylamine was given again. After a delay similar to the first latent period the blood sugar fell again rising later on the withdrawal of the thiol. Attempts were then made to achieve satisfactory control of the diabetes by adjustments to the doses of insulin and mercaptoethylamine but without success. During the subsequent period of trial with mercaptoethylamine the patient's course swung slowly from hyper- to hypo-glycaemia

* I acknowledge the kind co-operation of Mr E. M. Bavin of Smith & Nephew in supplying this compound.

† Equivalent to less than 1 gr in a 5 gr aspirin Aspirin did not influence the glycosuria in this patient

from week to week the excursions getting gradually larger On occasions she passed over 250 g of *glucose/day in the urine* only to pass several days later into a period of severe hypoglycaemia the control of which once required 1100 g of carbohydrate over a period of 24 hours

Many months later this patient was controlled by sulphonylurea treatment suggesting that she was capable of producing her own insulin

One was tempted to regard the initial aspects of the mercaptoethylamine study as evidence that the monothiol which showed a delayed action in contrast to the dithiol had *required metabolic* alteration to exert its effect However a full explanation of the extraordinary course subsequently shown by this patient is not possible at present The powerful metabolic action of the thiol could be due to some relationship between diabetes and thiol metabolism or to an action releasing insulin from binding substances in the blood Trials of mercaptoethylamine in other diabetics have *not been encouraging*

3 HYPERPYRUVATAEMIA IN ESTABLISHED DIABETES

Another feature of the initiation of a diabetic state in the burned patients was the occurrence of hyperpyruvataemia after glucose and insulin It was decided to test established diabetes in the same way seeking evidence for a similar hyperpyruvataemia which might be a residuum of the initial metabolic disturbance

To date over fifty diabetics have been investigated Abnormally high pyruvate levels have been found in the venous blood of about a third and especially high values occur in the severe poorly controlled (brittle) diabetics

These results were presented recently at the International Diabetic Congress by Dr Kelsey Fry Professor R H S Thompson and myself[5] and I will only summarize them here

We find that brittle diabetics have a pronounced rise in blood pyruvate during the first hour of a glucose-insulin tolerance test (see Table IV)

Normal subjects and most diabetics show a modest rise of *0 3 and 0 4 mg per cent* respectively during this period whereas values of up to *3 4 mg per cent* with a mean rise of *1 7 mg per cent* have been recorded in the brittle cases values *which are comparable* to those found in the diabetic burns

However we are now in a position to offer a little additional information about these patients Measurements of A-V differences across the forearm muscles in conjunction with blood-flow estimations make it possible to assess the peripheral utilization of pyruvate during a glucose-insulin test Preliminary data show that the arterial level of pyruvate may be considerably in excess of the venous level indicating that there can be peripheral utilization of pyruvate indeed during this phase there is negligible uptake of glucose and it might be argued that the tissues have a slight compensatory mechanism by introducing carbohydrate into the cells as diffusable 3-carbon pyruvate rather than as 6-carbon glucose!

TABLE IV

MEAN RISE OF PYRUVATE DURING GLUCOSE-INSULIN TOLERANCE TEST (MG) (100 G BLOOD)

	Controls	Diabetics (brittle)	Diabetics (other)
Rise at 60 min	0 3	1 7	0 4

The main implications of the findings of high arterial pyruvate levels are firstly that the pyruvate is probably elaborated in the splanchnic area and secondly that in our previous studies peripheral uptake by muscles could have masked the release of larger quantities of pyruvate by the splanchnic area than was suspected during the glucose insulin test Thus faulty pyruvate metabolism may be present in a larger proportion of the diabetic population than is at present recognized and the incidence of hyperpyruvataemia on the arterial side must be sought

Whether the hyperpyruvataemia found in these cases of established diabetes reflect a similar abnormality to that obtaining in the temporarily diabetic burned patients cannot be answered However we have reported that like the diabetic burned patients their glucose tolerance appears to be improved by BAL given for 3-4 days[4] These findings are in harmony with those of Mozo[15] and Asper and his colleagues[16] who have all reported improvement of the diabetic state during treatment with BAL.

In view of the previous evidence we have been tempted to speculate on the possibility that these patients suffer from some

disorder of thiol metabolism perhaps a relative deficiency of α-lipoic acid in the splanchnic region which was corrected temporarily by the injections of BAL [14]

4 CONTROL OF TWO YOUNG UNSTABLE DIABETICS WITH BAL ON AN OUT-PATIENT BASIS

To examine the role of BAL in therapy two young unstable brittle diabetics who responded to BAL for a few days in hospital were treated in conjunction with Drs C Hardwick and C L Joiner on an out-patient basis

The first case was a 26-year-old woman who had been diabetic since the age of 17 Her insulin requirements had gradually risen over the years and she was uncontrolled on 100 units insulin/day when the present study began Her blood pyruvates were elevated after glucose and insulin When she was tested with BAL in hospital her glycosuria diminished over the last day on the dithiol and her pyruvate levels during the glucose-insulin test fell On the withdrawal of BAL her diabetes returned to the previous uncontrolled state She returned home and was kept under close surveillance as an out-patient Her urine was tested before meals and recorded four times a day for 3 months the majority of these tests showed glycosuria (Table V) Insulin injections were then augmented with

TABLE V

EFFECT OF BAL ON GLYCOSURIA DURING OUT PATIENT STUDY ON 6-YEAR-OLD DIABETIC UNCONTROLLED ON 100 UNITS INSULIN/DAY

Test result	*Frequency on 100 units insulin/day (3 months)*	*Frequency on 100 units insulin/day and BAL (3 months)*	*Statistical analysis (2×2 contingency)*
>2 per cent	21 per cent	0 5 per cent	P< 001
2 per cent	23 per cent	0 5 per cent	P< 001
1 per cent	40 per cent	7 per cent	P< 001
½ per cent	10 per cent	per cent	P< 001
0 per cent	6 per cent	70 per cent	P< 001

BAL 200 mg as 5 per cent solution in oil i m three times/week urine testing continuing Over the first month there was diminution of the glycosuria which was maintained By the end of 2 months the majority of urine tests were negative for sugar (Table V) During this period the patient had a few episodes of moderate

hypoglycaemia she also gained weight. Unfortunately after 3 months albuminuria was detected This was attributed to the BAL Renal biopsy was performed no microscopic abnormality was detected BAL was then stopped The albuminuria cleared up within a week and the diabetic control was gradually lost A subsequent course of BAL was also associated with improved control but albuminuria recurred It was deemed inadvisable to continue this therapy further

Similar findings were made in a 20-year-old male patient with unstable diabetes who also had high levels of pyruvate especially in arterial samples during the glucose-insulin test His out patient regime before BAL included 130 units insulin/day During the control period before BAL the majority of the urine tests performed before meals indicated heavy glycosuria

BAL was given -00 mg 5 per cent in oil three times/week There was a clear-cut reduction in the number of glycosuria urine samples over the subsequent - months of treatment Statistical analysis again indicated that the reduction of incidence of glycosuria was statistically significant The patient suffered several hypoglycaemic attacks and his insulin dosage had to be reduced to 100 units/day However he too developed albuminuria and the thiol had to be discontinued Withdrawal of BAL was followed by a reversion to the previous uncontrolled diabetic state without albuminuria We attributed the albuminuria to the kelating effects of BAL on trace metals involved as enzymatic co-factors in the kidneys As the patients gained weight on BAL its action was not due to interference with glucose absorption Nevertheless we must conclude that BAL offers no prospect as a therapeutic agent for the long term control of unstable diabetes

Conclusion

In each of the four investigations reviewed here the facts may be reconciled with a general hypothesis that diabetes may frequently be associated with an abnormality of thiol metabolism However such a reconciliation does not mean that the explanation offered is necessarily correct It is salutary to recall Hamlet s exhortation – There are more things in heaven and earth Horatio than are dreamt of in your philosophy

REFERENCES

1 ANDERSON G E WIESEL, L L HILLMAN R. W & STUMPF, W M (1951) *Proc Soc Exp Biol N Y* 76 825
2 BUTTERFIELD W J H (1954) *Proc Roy Soc Med* 47 228
3 BUTTERFIELD W J H (1955) *Lancet* i 483
4 BUTTERFIELD W J H & THOMPSON R. H S (1957) *Cl n Sci* 16 679
5 BUTTERFIELD W J H KELSEY FRY I & THOMPSON R. H S (1958) Proc 3rd International Diabetic Congress, Dusseldorf
6 CONN N W LOUIS L H & JOHNSTON M W (1949) *Science* 109 279
7 CORCORAN A C & PAGE I H (1948) *J Lab & Clin Med* 33 1326
8 EVANS E I & BUTTERFIELD W J H (1951) *Ann Surg* 134 588
9 GITELSON S (1954) *Acta Endocr* Copenhagen, 15 225
10 HOET J P & LUKENS F D (1954) *Diabetes* 3 1
11 JACKSON W P U & WOLFF N (1957) *Lancet* i 614
12 KERPPOLA W (1953) *Acta Med Scand* 145 357
13 LAZAROW A (1954) *Exper mental Diabetes* Ed J P Hoet and F G Young Blackwell Scientific Publications Oxford p 49
14 LONG D A MILES A A. & PERRY W L M (1951) *Lancet* ii 90
15 MOZO M R. Doctoral Thesis (1952) Los problemas de acids urico y del azurfe en al Diabetes Mellitus Seville
16 PROUT T E WEAVER J A SCOTT G W & ASPER, S P JNR (1958) *Metabol sm* 7 240

A COMPARISON OF THE MECHANISM OF ACTION OF INSULIN WITH THAT OF THE HYPOGLYCAEMIC SUBSTANCES

R Levine

Department of Medicine Michael Reese Hospital
Chicago 16 Illinois

Any French accent which you may detect comes from the laboratory in Montpellier since I am substituting for Professor Loubatieres

When Professor Young asked me late last night after several assorted alcoholic beverages to do this job I did not have the strength of character to resist He expected that I should have (and was disappointed that I did not have) any slides with me

Now to the subject matter itself The blood-sugar lowering effects of certain sulphonamide derivatives was noticed sporadically some few years before the fundamental discovery by Jambon and Loubatières No attention was paid to it because it was considered to be a toxic side effect But as you well know Jambon noticed the intense hypoglycaemia after the administration of isopropylthiodiazole in 1942 Immediately thereafter Professor Loubatieres at Montpellier undertook the physiological and pharmacological study of this and other closely related compounds and he did a magnificent job in the 4 years between 1942 and 1946 We must cast our glance back to those years and remember that a war was raging at that time We must consider the difficulties under which these papers were published because they were published in 1946 They received little notice except from a few isolated individuals namely La Barre in Belgium and Chen in the United States A few years later the hypoglycaemic property of another sulphonamide (known as BZ 55) was discovered in Germany and soon after the effects of its methylated derivative — tolbutamide The pharmacological work in Germany took at first an entirely different direction from that of Loubatieres The clinical work began almost immediately and actually we had few pharmacological data about these compounds at that time after several hundreds of patients had already been

treated I should like to remind you that by 1946 Loubatieres had established for IPTD (isopropylthiodiazole) certain experimental facts which have since then been abundantly confirmed also for the newer sulphonylureas

The experimental animals were dogs and rabbits In the normal dog one could lower the blood sugar with sulphonylureas If the animal s pancreas were completely removed the acute administration of the sulphonylureas by mouth or by any other route no longer produced lowering of the blood-sugar level If the pancreas were removed only partially, the blood sugar would fall Loubatieres therefore called these substances pancreatotropic and considered that they tended to liberate insulin from the β cells About one-sixth of the pancreas of the dog was needed to produce a brisk hypoglycaemia *It was also established that nerve pathways* were not concerned in the production of hypoglycaemia (The spinal cord the splanchnic nerves and the vagi) The endocrine glands other than the pancreas were not necessary for the demonstration of the IPTD hypoglycaemia The glands were excised one at a time in the same animal Neither thyroidectomy castration adrenalectomy nor hypophysectomy *singly or jointly interfere* with the blood-sugar lowering effect The final operation r of the pancreas abolished the action

The effects of these drugs were then tested in allo zed Alloxan diabetes of course is a variable state since one may varying degrees of destruction of the β cells The α cells are tained in normal histological state in alloxan diabetes In severely alloxanized rabbit these drugs did not lower the sugar However if there was only a partial alloxan diabetes the blood sugar was lowered despite the fact that the α cells intact This is important because some time later studies of von Ferner and others implicated a depression of the α cells *therefore presumably a depression of glucagon manufacture* secretion as the reason for the hypoglycaemia This view present no real support even by its original proponents

Loubatieres s early work showed therefore that the requisite for the acute hypoglycaemic action of the is the presence of a sufficient number of normal β cells *pancreas The α cells do not* play any significant role in this

The success of the clinical testing of the sulphonylureas last few years has spurred on a large volume of research

intimate nature of the mechanisms of action We shall now present some of the salient features of the general results in this field Let us examine first the possibility of direct action on the peripheral tissues from the results obtained in experiments done on liverless and eviscerated animals and on isolated muscle fat or such other tissues which are normally insulin sensitive with a view to determining whether the sulphonylureas imitate in any way the action of insulin With very few exceptions the consensus of opinion at this moment is that by themselves the sulphonylureas do not increase the utilization of glucose do not change the oxygen consumption or CO_2 production and do not lead to a distribution of the non-utilizable insulin sensitive sugars They therefore in no way imitate the action of insulin

From the previously cited evidence of Loubatieres which more recently was confirmed and extended by Professor Houssay it is evident that sulphonylurea hypoglycaemia is not dependent on the inhibition of contra-insulin factors in the pituitary adrenal etc

The importance of the β cell was substantiated in man Diabetes developing after surgical pancreatectomy was not influenced by these drugs Juvenile diabetes in which Wrenshall has shown that the pancreas possesses a very low insulin content cannot be treated with these drugs However adult onset diabetes of the obese non-ketotic variety in which pancreatic insulin content tended more towards the normal values can be treated both acutely and chronically by sulphonylureas showing the importance of the β cell in the response of human diabetes mellitus

Houssay Caren and others since have observed that under certain conditions in the depancreatized animal when the dose of insulin is small and the dose of sulphonamide is rather large one can see a potentiating effect in the absence of β cells but in the presence of exogenous insulin These findings disturb the unitary hypothesis that the action of sulphonylureas is only on the β cell

If insulin is indeed the mediating agent in the action of the sulphonylureas either by stimulating the β cells to secrete insulin or by potentiating it peripherally or both then one should see in the animal or man given the sulphonamides the same metabolic effects one gets after insulin Many of these have been looked for and the literature on the subject is most confusing and contradictory A rise in pyruvate occurs after insulin and glucose administration After tolbutamide despite the lowering of the blood sugar the rises in

pyruvate were not obtained by some workers and were seen by others An increase in A-V differences of glucose in the peripheral limbs was obtained by some and not seen by others I think Dr Butterfield is numbered among those who did obtain them with tolbutamide if I am correct

It was supposed that if a certain amount of insulin lowered the blood sugar by 40 mg per cent and if one gave tolbutamide to lower the blood sugar an equal 40 mg per cent one should expect exactly the same results in all other blood constituents If indeed the tolbutamide acts by releasing insulin from the pancreas then it would release it not in 10-unit amounts as if from a syringe but rather in very very small amounts at a constant rate over a certain period of time Also the liberated insulin would enter the portal system and not a peripheral vein Thus the insulin could act on the liver before it came to the periphery or a large portion of the insulin could be destroyed by insulinase in the liver itself

One of the earliest workers to cast doubt on the neat picture of a pure insulin effect of the sulphonylureas was Dr Renold who did a simple experiment it is always the simple experiments whic are bothersome He showed that when one gives fructose to diabetic the blood glucose rises as a result of transformation in liver When tolbutamide was given the glucose rise was pointing to an effect perhaps an inhibition of liver sugar *We therefore come full tilt up against the problem of the* of the sulphonamides not in the periphery but upon the This problem has two facets and these two facets can best characterized by calling them the evident aspects of the phrenia of the subject If one holds that the sulphonamides st the β cell that insulin is then released via the portal system reaches the liver and if the sulphonamides have no other ac than this then there arises the contradiction that the o sugar by the liver seems to be inhibited by the sulphonamides but not by the direct infusion of insulin

Weinhouse *et al* using ^{14}C glucose find that the output o by the liver is inhibited by both the sulphonamides and However Schambye s data are the same as those of the group namely that the sulphonamides inhibit sugar output liver but that insulin does not

The sulphonylureas do not lower the blood sugar of an from whom both the liver and pancreas have been removed

ever if the pancreas is left in the abdomen (by porto-caval anastomosis) when the liver is removed and the blood-sugar level is maintained by the constant infusion of glucose the sulphonylureas *will produce hypoglycaemia Thus the liver is not an indispensable* organ for the hypoglycaemic effect That does not mean however that when the liver is in that the tolbutamide does not have a direct or indirect action on the liver as well

The evidence for the liberation of insulin from the pancreas by the sulphonylureas is quite good especially when one adds to that the work of Pfeiffer (done with the Harvard group) in which the injection of tolbutamide increased the plasma insulin level especially in the portal system This view does not explain the potentiating action in the depancreatized animal A few years ago when we were dealing with these confusing aspects Dr Krahl suggested that insulin may combine with a tissue component and thus remain inactive *and that the sulphonylureas split this complex They would* then liberate insulin from the complex and make it available Such an action may occur in the β cell but also in the peripheral tissues which would account for the pancreatic as well as the potentiating effect

Since the title included other oral hypoglycaemic agents I should like to comment shortly about another type that is at present under clinical and experimental trial namely DBI The biguanides chemical relatives of synthalin seem to act in the absence of the β cell on peripheral tissues e g on muscle *in vitro* They stimulate like Dr Randle s dinitrophenol and other depressants of cell respiration the uptake of sugar by the cell The action resembles the state of partial *anaerobiosis and just as in anaerobiosis the lactic acid in* the blood increases tissue glycogen falls and some of the oxidative systems of the cytochrome series are inhibited We are dealing with an inhibition of oxidative metabolism and a consequent greater anaerobic utilization of glucose under these circumstances

It is contended on the basis of A-V differences done over a short time period that as a result of tolbutamide action there is *no* increased utilization of carbohydrate It is however impossible to maintain such a conclusion for the following reason Suppose that a 50-year-old adult obese diabetic is excreting 30 to 50 g of sugar in the urine and requires for control about 30 units of insulin per day and eats a diet of 1500 to 1800 calories per day including 150 g of carbohydrate He is then placed on tolbutamide and maintained

on it for 1 or 2 years At the end of that period he is well and healthy has been working satisfactorily has no glycosuria is not emaciated and his fat depots are normal It would seem that all of the dietary carbohydrate (365 × 250 g per year) must have been utilized in a perfectly normal manner in such an individual

REFERENCES

1 LEVINE R. (1957) *Annals N Y Acad Sci* 71 1
2 LOUBATIÈRES A (1958) *Presse Medicale* 66 1175
3 LOUBATIÈRES A (1958) *Presse Medicale* 66 1229
4 Symposium on Sulfonylureas (1956) *Metabolism* 5 721
5 Symposium on Tolbutamide (1956) *Deutsche Med. Wochschr* 81 823

DISCUSSION

LONG I was particularly interested in Dr Ginsburg s failure to demonstrate any peripheral effect of hydrocortisone even though it was given in very large amounts over a relatively short period of time It has been our experience using animal preparations — eviscerated nephrectomized rats in fact — that it is not possible to demonstrate any effect on either the utilization of glucose by these animals or any modification of the response to insulin when large amounts of hydrocortisone are given This of course contrasts very sharply with the ready demonstration of the effect of the same hormone on the carbohydrate metabolism of the liver and I think emphasizes what I hinted at yesterday that in all probability the main effect of the adrenal steroids is directed towards the carbohydrate metabolism in the liver and that any effects that may occur in the peripheral tissue are probably secondary to this For example in the so called steroid diabetes it can be shown that first effects which follow the administration of hydrocortisone are a great accumulation of glycogen in the liver This is followed at a much later period by an increase in the muscle glycogen undoubtedly due to the fact that at the time when the liver glycogen has risen to these high levels the blood sugar has also risen One other point in relation to the adrenal cortical hormones and this question of the demonstration of glycogen synthesis particularly in liver slices is that it has been our experience in adrenalectomized animals that the ability to retain or perhaps form glycogen in the liver from a variety of precursors is very considerably diminished in the absence of the adrenal corticosteroids I have been wondering whether in many experiments where attempts have been made to demonstrate *liver glycogen synthesis as for example in slices* by the addition of insulin this will be possible unless cognizance is taken of the important role played by the adrenal steroids in the formation or perhaps we should even say the retention of glycogen in the liver

STEWART Following on Dr Wolff's paper I would like to make one or two observations

Firstly Madison and Unger recently described the effects of administering insulin endoportally and peripherally and found the distribution of the insulin was markedly different By the endoportal route 51 per cent was found in the liver and 27 per cent in muscle and by the peripheral route the ratio changed and 56 per cent was found in the muscle and only 27 per cent in the liver The A-V glucose difference was smaller after endoportally administered insulin than after peripherally administered insulin We believe that we are stimulating the release of endogenous insulin and therefore some of the differences in our experiments within a group may possibly be explained by the way in which the endogenously released insulin is distributed throughout the body of each subject

Another point is the question of the diabetics who are non ketotic I remember Professor Young saying a short while ago that he had seen some dogs which were rendered diabetic by pancreatectomy that the administration of a minute amount of insulin removed the evidence of ketosis without affecting the blood sugar level and that too maybe has some bearing on these differences

Fig 1 shows the effect of administering both carbutamide and tolbutamide to groups of guinea pigs and determining the maximum fall in blood sugar within these groups The third group which received insulin is also shown The main point I want to bring out is that with the sulphonylureas there is a dose beyond which as it is increased the amount of hypoglycaemia gets less and although these doses in animals are much larger on a body weight basis than those given to man Dr Wolff and I and others have shown that in man this sort of effect can take place with much smaller doses It may be due to a stimulation of the adrenals as Bander has shown

Fig 2 shows the effects of administering glucose and tolbutamide to two groups of rabbits The groups on the right are rabbits which have at one time been rendered alloxan diabetic and then allowed to recover in so far as their blood sugar level came back to the normo-glycaemic range When tolbutamide is administered to these rabbits there is during the first 2 hours no evidence of hypoglycaemia compared with the response in the normal rabbits When glucose is subsequently given the tolerance of these animals which have shown a remission of the alloxan diabetes is less than that of the normal animals Interestingly enough the rabbits which have shown this remission of alloxan diabetes have histologically normal β cells of the pancreas according to my colleague Dr Udall and also their insulin content can be shown to be normal by bio-assay So it may be that the suggestion Dr Wolff made that tl

sulphonylureas may act on some specific mechanism in the pancreas to cause the release of insulin may be an important factor in their mode of action.

HOET In relation with the mode of action of hypoglycaemic sulphonylurea, I wish to draw the attention to experiments done by de Meyer and M Isaac-Mathy about the teratogenic action of BZ 55

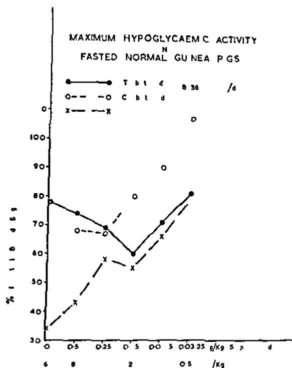


FIG 1 The effect of dose on the maximum hypoglycaemic response of fasted normal guinea-pigs to carbutamide tolbutamide and insulin (uphonamides administered orally insulin subcutaneously)

When a single peroral dose of 500 mgms is given between the ninth to the eleventh day of pregnancy the rat delivers newborn who are mal formed oedematous and in a vast majority anophthalmic This is *absolutely different from the pictures* of some bone abnormalities seen after profound and repeated hypoglycaemic convulsions With BZ 55 *the pregnant rat has no symptoms* but the offspring has lesions of the eyes and the optic nerve Another sulphonamide — not hypoglycaemic

did not result in any teratogenic effect As long as this teratogenic effect is not clearly understood and as long as we are not absolutely certain tha side effects on the foetus are not harmful it seems advisable not to give BZ 55 to pregnant women not even when the carbohydrate disturbance is mild (de Meyer et Isaac Mathy A propos de l action teratogene d un sulfamide hypoglycemiant (N sulfanilil N nutylurée — BZ 55) *Les ann d Endocr* 1958 XIX 1 168-7.)

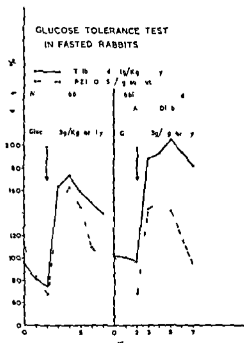


FIG 2 The tolera ce of n rmal abb ts and of abb ts which ha e reco ered *from* alloxan diabetes to glucose 2 hours after they hav received either tolbutamide or prot mine zinc insulin (PZI)

YOUNG I am sure you will all agree after hearing Dr Levine s remarkable dissertation this afternoon that I was right in my judgment last night in approaching him at whatever stage of the evening it was to ask him to deputize for Professor Loubatières I am sure that this paper was not the only one for which Dr Levine could have effectively but he certainly did brilliantly on this occasion

Likewise I hope you will agree that it was not unreasonable for me to expect that he might have slides on him In fact I should not have been surprised if he had had slides with the captions in French! He is prepared for almost anything If I may follow Professor de Duve s example and use a French expression I will say that he met the invitation to deputize for Professor Loubatieres with complete *sang froid*

I would like to mention some experiments with sulphonamides that we have had under way in Cambridge for some years Miss Jeacock is collaborating with me in this work which is necessarily long term We have been treating cats that have been made persistently diabetic by a short period of treatment with growth hormone (metahypophyseal diabetic cats) with anti-diabetic sulphonamides I should say that these cats will show in all instances in which they have been followed long enough spontaneous remission from this diabetic condition sometimes after a period of a year or more of untreated diabetes The glycosuria may be heavy indeed and yet ultimately the glycosuria disappears and the blood sugar becomes normal

In the few experiments we have been able to do on animals of this sort the administration of anti-diabetic sulphonamides has caused the diabetic condition to clear up much more rapidly than it would have done spontaneously and the problem arises as to what is the mechanism involved Are these animals diabetic because there is a deficient secretion of insulin by the pancreas? If that is true what has the sulphonamide done? Has it unleashed some inhibiting point in the production of insulin in the pancreas of these animals? We have no very clear ideas on this point I do not know whether Dr Levine has any ideas in this connection

I would like to make a comment too while I am here Mr Chairman about some remarks that Mr Stewart made earlier concerning experiments of my own on the effect of small doses of insulin on ketonuria I referred to these experiments at a meeting some time ago they were done more than 20 years ago and were concerned with depancreatized dogs which were being investigated as control animals for metahypophyseal diabetic dogs I was impressed that the metahypophyseal diabetic dogs showed a very substantial glycosuria but no ketonuria I was able to show that the administration of a very small dose of insulin to depancreatized dogs could completely abolish the ketonuria without any significant effect on the glycosuria or D/N ratio I could not say that there was no temporary fall of blood sugar level in these animals given the small doses of insulin that we were using but certainly there was no discernible effect on the total magnitude of the glycosuria over 24 hours nor on the D/N ratio I am not quite sure just what is the significance of those experiments but I think the experimental findings were valid

STOWERS I have two questions to put to Dr Reid The first is this

Does he need serum salicylate levels as high as 35 45 mg /100 ml to achieve satisfactory hypoglycaemia? Our own work on a relatively small scale indicates that serum salicylate levels of 10-20 mg /100 ml may be associated with good hypoglycaemia

The second question is about the B M R effect of salicylates Does this persist or is it rather transient associated with the loss of intracellular energy stores and the potassium already mentioned? Preliminary results which we have obtained suggest that the B M R effect is transient and not necessarily correlated with the hypoglycaemic effect

REID In answer to Dr Stower s first question it is not necessary to maintain high salicylate levels to obtain an effect in blood sugar but we think it is necessary to get a maximal effect and this is what we are trying to find out though it is too soon yet to say what the ideal level is In reply to the second question the metabolic stimulation shown by increased oxygen consumption is not transient but is directly related to the serum salicylate level In other words a reduction in oxygen con sumption is in our experience invariably accompanied by a fall in serum salicylate level even though dosage is unchanged This does not happen in all patients but it emphasizes the need to control therapy by estimation of serum salicylate level particularly in the early stages of treatment We have no information at present on the relation between the hypoglycaemic effect and increased oxygen consumption induced by salicylate

LEVINE Since Professor Young raised the question I feel duty bound in a sense since I am substituting for Professor Loubatières to answer as I think he would There is one mode of action which he has hinted at even in 1946 and 1947 and lately since 1955 That is the effect that the sulphonamides may have on the quick recovery from mild alloxan diabetes Something analogous perhaps to the quicker recovery from metahypophyseal diabetes Perhaps the sulphonamides in a partial damaged pancreas act to stimulate the new formation of β cells the duct epithelium

I wonder Professor Young if you have some histological sections comparable animals treated and untreated with sulphonamides to see that notion is perhaps correct

YOUNG I am afraid we have nothing clear-cut on this point at

BESSMAN I should like to call attention to some work that has done and repeated by a number of people now initially by Fried then by Horwitt and, recently the very elegant studies of H the *Journal of Clinical Investigation* of the last 6 months on the measuring the blood pyruvate alone as an index of metabolism of thing whatsoever The blood pyruvate is remarkably closely the blood lactate and the blood lactate is affected even by In fact, you can raise the serum lactate concentration by

3 to 4 milli-equivalents by hyper-ventilation Therefore results obtained with pyruvate levels in patients particularly those treated with salicylate which is known to cause a hyper-ventilation alkalosis as part of its toxic effect could hardly be interpreted as carbohydrate effects

REID There is one point about salicylate s action in diabetes that might be amplified We have shown that the more easily a patient is controlled with insulin the better the response to salicylate Furthermore there seems to be an increasing response to salicylate as insulin requirement rises This may have a useful practical application in patients requiring large daily doses of insulin who are troubled by hypoglycaemic attacks Combined aspirin-insulin therapy by drastically cutting insulin requirement may also reduce the frequency of hypoglycaemic attacks

BUTTERFIELD Although I am acquainted with the experiments referred to by Dr Bessman about pyruvate metabolism, I would like to point out that none of my patients was on salicylates or hyper ventilating or getting alkaline infusions The pyruvate changes I described are regularly reproducible and seem to reflect metabolic changes other than the factors Dr Bessman mentioned

Turning to the mode of action of the sulphonylureas I believe there has been a good deal of confusion about interpretation of peripheral A V glucose differences measured before and after treatment with these compounds I hope we shall be able to publish shortly our findings in some experiments measuring peripheral glucose uptake The results suggest a simple explanation which seems to me to clear up many of the difficulties (Effects of insulin, tolbutamide and phenethyldiguanidine on peripheral glucose uptake in man *Diabetes* 1958 7 449)

PART IX

CLINICAL ASPECTS OF DIABETES AND PRE-DIABETES

Chairman PROFESSOR F G YOUNG

SOME CLINICAL ASPECTS OF DIABETES WITH RELATION TO INSULIN AND TOLBUTAMIDE

W P U JACKSON

Department of Medicine Cape Town

VARIETIES OF DIABETES

I should like to start by reaffirming the clinical s paration of diabetes mellitus into three types severe mild and secondary Severe refers to the usually young growth-onset ketosis-prone individual who needs insulin to live Mild refers to the usually older maturity-onset often obese person who does not become ketotic when insulin is omitted although he (or usually she) may be relatively insensitive to insulin and require 100 or even more units to produce any obvious effect on her carbohydrate metabolism The dose of insulin being used is no criterion of the severity of the diabetes Severe and mild diabetes are sometimes called pancreatic and extra-pancreatic respectively on the assumption that the di.. ease in the first case is due to destruction of pancreatic β cells and the second to anti insulin factors which may or may not be monal However insulin antagonists have been found in the blo stream of the severe diabetic rather than in the mild case wl islet-cell damage probably always occurs in the pancreas of the diabetic in whom both Ogilvie[5] and Gepts[1] have found an inv... reduction in amount of islet tissue Vallance-Owen[7] has shown the insulin-like activity in the blood stream of the mild d of the same order as in a fasting normal person This hc does not mean that the pancreatic activity is normal – far since an induced ri e in blood sugar in the normal to the that in the mild diabetic produces a several fold increase in insulin activity Thus the mild diabetic possesses a pancreas is unable to respond normally to the stimulus of a raised level

It may be noted that the terms mild and severe refer the evidence of abnormality of metabolism – both develop the same crippling or killing vascular compl may present similar inheritance patterns and occur togetl

same family and both may give evidence of their latent existence by the same sort of abnormal obstetric history and embryopathy in the pre-diabetic phase It is also true that a mild diabetic may develop ketosis under the influence of certain forms of stress while occasionally a severe diabetic reverts to the mild form I have twice

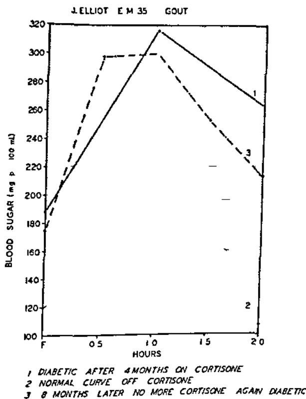


FIG 1 True diabetes uncovered by cortisone

seen patients in hospital in diabetic coma without any known precipitating factors who normoglycaemic some months later without insulin Nev clinical dif of the main primary types of remarkably often neglected For use of sulphonylureas with of dosage and

so on are quite useless unless the two diabetic groups have first been clearly separated

Secondary diabetes refers most obviously to that which follows chronic destructive pancreatitis haemochromatosis or total pancreatectomy The diabetes which occurs during pregnancy staphylococcal infections acromegaly or moderate doses of glucocorticoids is not truly secondary but rather the unmasking of an underlying latent or pre-diabetic state The first figure shows a corticoid diabetic becoming apparently normal after cessation of treatment but again diabetic – this time permanently – a few months later

PRE DIABETES AND VASCULAR DISEASE

Many of us believe that strictly speaking diabetes begins at birth that the basic abnormality whatever it may be is present for the whole of the lifetime of the diabetic although the overt carbohydrate defect may not be manifest until middle age Time precludes discussion of the arguments in favour of this belief beyond my remarking that the elderly diabetic who attends your clinic may give a history of large babies and stillbirths which extends back 30 40 or 50 years into her pre-diabetic past Furthermore we consider that the specific vascular abnormalities of diabetes are not complications but are an integral part of the syndrome inherited together with the metabolic disturbances Usually they appear later than the hyperglycaemia but sometimes before it They are not a consequence of hyperglycaemia and normalization of the blood sugar even in the very mildest cases is no safeguard against their occurrence It would however appear that they are closely connected with pancreatic damage since true secondary pancreatic diabetes has occasionally led to the later development of specific retinopathy or nephropathy

These various observations are extremely important since they appear to intimate that a defect of pancreatic islet tissue may produce a diabetic vasculopathy which is not dependent upon hyperglycaemia Indeed we have perhaps been over-obsessed with hyperglycaemia as the primary feature of diabetes Insulin has an action in fat metabolism which may possibly be more important than its more obvious effect on carbohydrate It is closely related to the level of the non-esterified fatty acids and stimulates rapid metabolism in fatty tissue It has now been shown to exert an influence

on amino-acid metabolism[8] Might it perhaps have an essential protective effect on blood-vessel walls? A future diabetic might be born with a relatively inefficient β-cell mechanism not so defective that our rough yardsticks of definition based on glucose tolerance can detect it but bad enough so that throughout the years certain blood vessels in certain places become progressively damaged The special stress of pregnancy may indicate the latent deficiency by producing temporary carbohydrate intolerance in the mother death or excessive size of the foetus and hyperplasia of the foetal islets of Langerhans

INSULIN AND PRE-DIABETES

This hyperplasia of the islets of Langerhans in the foetus of a diabetic or a pre-diabetic mother is a remarkable phenomenon Fig 2 shows the relative proportions of islet tissue to whole pancreas

MEAN ISLET AREAS OF STILLBIRTHS

CONTROLS	1 3%
DIABETIC MOTHERS	6 5%
PRE DIABETIC MOTHERS	7 5%
ERYTHROBLASTOTIC BABIES	7 1%

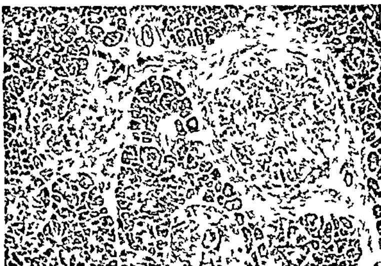
NOTE SQUARES INDICATE RELATIVE SIZES ONLY

Fig 2 Mean proportion of islet tissue in pancreases in different groups

which Woolf and I[9] found on examination of 108 pancreases from stillborn infants Fig 3 illustrates a particular pancreas from a foetus of a pre-diabetic mother compared to a normal at the same magnification From the small amount of suitable material available to us it seems that not only are the islets large and increased in number but they contain an unusually high proportion of β cells and these β cells contain an unusually great concentration of granules If granules really represent insulin then it would appear that the pancreas of such a foetus may contain up to thirty times as much insulin as normal What could be the cause of this β-cell hyperplasia? It is not hyperglycaemia because it occurs in the absence of hyperglycaemia in the pre-diabetic Growth hormone and gluco-



[illegible]



[illegible]

corticoids have been much discussed in connection witl embryopathy but there are cogent arguments agai these being the sole or primary villain of the piece ? maternal lack of pancreatic reserve during pregnar through some mechanism other than hyperglycaemia compensatory hypertrophy of the infant s islets? Now th insulin produced by the foetus might itself act as a growth ' in its own intra-uterine development Could this possib' for the excessive size of the diabetic s baby?

If this story has any truth then insulin might be - p prevent the diabetic vascular abnormalities the foetal and abnormalities and possibly the actual development of overt in a pre-diabetic Certainly and unfortunately insulin will nc vent all vascular disease in diabetics but there is much eviden when it is given in such a way that good control of glucose bolism is achieved then the liability to vascular lesions is in considerably lessened Figures from our own clinic indicate the incidence of retinopathy and neuropathy in poorly con diabetics was double that in diabetics whose control was goc excellent Now if our belief regarding the presence of diabetes fr birth is true it is surely plain that insulin given only when ov carbohydrate defects are present would not be expected to prev a lesion which has already been developing for a long time If v could give insulin early in the pre-diabetic phase might we not prevent the vascular disease This of course is not known but is worth considering Methods used in diagnosing pre-diab I will not consider here but it may be mentioned that certain family histories make a diagnosis of pre- or latent diabetes mandatory for instance when both parents are diabetic when an identical twin is diabetic or in such a family as is shown in Fig 4 Professor Hoet[7] has indeed some evidence that insulin given to a pre-diabetic woman during pregnancy will prevent the development of severe congenital abnormalities while Wilkerson[8] from Boston has shown that the same treatment will tend to reduce the birth weight of the foetus. Further Hoet has pointed out that the child of a known diabetic mother being treated with insulin seldom becomes diabetic under the age of 10 whereas almost all mothers of children who become diabetic under the age of 6 are untreated latent or pre-diabetics Insulin given during pregnancy may thus have a protective action on the child Is it just possible that insulin given to

children in diabetic families would prevent diabetes entirely? It protects the pancreas of animals against alloxan

PROPHYLAXIS WITH INSULIN OR ORAL SULPHONYLUREAS

It is admittedly impracticable to suggest that babies or even adults should be stuck with a needle every day without a great deal more evidence of its value Could the oral sulphonylureas take the place of insulin in prophylaxis? It is very important that we know the mode of action of these substances before embarking on such a programme If their major action is a stimulation of β cells with th

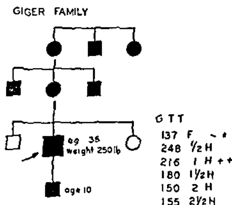


FIG 4 The blacked in figures represent diabetics The proband (arrowed) had a diabetic son mother and grandmother There was no diabetes on his wife's side He did not know he was diabetic but the family history led to his having a tolerance test result as given.

production of insulin then there are two ways in which their use in prophylaxis would differ from that of insulin First it might be presumed to overtax the already inefficient β cells rather than to rest them The whole idea of continued stimulation producing permanent structural damage to the β cells is however rather nebulous has been too readily accepted and finds no proven counterpart in the rest of the endocrine system nor indeed in other organs of the body Gepts[1] furthermore has found that the damage to islet cells in elderly patients treated with carbutamide or tolbutamide is no greater than that seen in a group of similar untreated or insulin-treated diabetics while in some he found actual regeneration of apparently active β cells The second difference

between tolbutamide and *exogenous* insulin is that the *endogenous* insulin which is formed under the influence of tolbutamide would be liberated into the portal system and so might really act more physiologically than insulin injected peripherally Thus Madison and Unger[6] have shown that insulin administered intraportally in dogs produces a much smaller arterio-venous glucose difference than when systemically introduced suggesting a lesser effect on muscle and probably a greater hepatic effect Tolbutamide does not appear to be toxic in other ways – in fact it seems to be about the most non-toxic drug ever produced Theoretically it might appear inadvisable to give it to a pre-diabetic or diabetic during pregnancy since we have evidence that the β cells of the foetus are already being excessively stimulated Although tolbutamide might be called simply a drug of convenience in the treatment of the established mild diabetic it may yet be of true value if used early enough especially in the pre-diabetic

The advent of the anti-diabetic sulphonamides has taught us more about diabetes as seen in the clinic In the trial of carbutamide and tolbutamide which we carried out we made it a rule to withhold all insulin from our mild diabetics for several weeks before starting the new drug in order to obtain a proper control baseline We were surprised to find a number of patients whose carbohydrate metabolism was as well controlled on diet only as it had been with insulin also In fact in a few the control was actually better without insulin This has led to a considerable reduction in the number of patients taking insulin in our clinic Furthermore it clearly indicates that any trial of an oral preparation in diabetes is valueless without periods during which no drug at all is taken Although insulin was apparently being given unnecessarily to the above-mentioned patients yet perusal of their previous records frequently indicated that it had been prescribed to good purpose when originally administered - 3 or 5 years before This might be interpreted in two ways either the disease in its natural course had become milder or as I prefer to think the insulin had really had a partially curative effect

Some clinics in this country have been strongly advocating a return to the use of soluble insulin alone for the control of all severe insulin-dependent diabetics They claim not only better carbohydrate control but also a greater protection against vascular disease than is afforded by long-acting insulins and in particular the

lente insulins This may be so in some clinics but it must be remembered that the long-acting insulins were introduced largely because two injections of soluble a day were inefficient in diabetic control We have found after some 5 years experience that the lente group of insulins are usually satisfactory and we consider that their wholesale abandonment would be a retrograde step unless more general evidence is forthcoming to warrant such a procedure

PRIME IMPORTANCE OF VASCULAR DISEASE

Despite the above arguments I do not wish to over-emphasize the potential protective value of insulin in diabetes Now that coma is a relatively minor problem the importance of diabetes does not lie in its carbohydrate control but in its vasculopathies – retinopathy neuropathy (assuming that this is basically vascular) nephropathy coronary and peripheral vascular disease The blood sugar of itself is a matter of little importance Although good control of diabetes as measured by glucose estimations may partially prevent the specific diabetic vasculopathies yet it has little or no effect on the development of coronary heart disease (Liebow *et al*[4]) We have observed this in figures from our own clinic Normally pre-menopausal women are considerably protected from the clinical manifestations of coronary atheroma by the very fact of their womanhood In this respect diabetes abolishes the advantage of being born a woman If however the coronary arteries can really be protected by a low intake of saturated fat then surely the very first place in which this type of diet should be advised is the diabetic clinic? If the carbohydrate intake has to be increased and so-called control rendered more difficult is there any evidence at all that this would matter? It seems to me that many 'diabetic diets being used today are probably atherogenic As recently as June 1958 a report of the Council on Food and Nutrition (of the United States) recommended that 40 per cent of calories in a diabetic s diet should come from fat Admittedly a small face-saving and rather ingenuous rider was added with unhydrogenated vegetable oils substituted to a considerable extent for hard cooking fats Comment on the utter *inadequacy of such advice should* be unnecessary There is certainly less evidence that the *specific* diabetic vascular lesions might be reduced by a lowered fat intake but this is something that must be investigated

I think that all this may be summarized by the suggestion that

insulin and even possibly the oral sulphonylureas may have a greater potentially protective action in diabetes than we realize but that drugs alone are not likely to solve the real clinical problem of diabetes – the vascular one We still do not know how best to treat diabetes and are only just beginning to think about preventing it

I should like to take this opportunity of thanking Professor J F Brock and Dr J H Sheldon for reading this manuscript

REFERENCES

1 GEPTS W (1957) Contr bution a l étud morphologique des îlots de Langerh n au cours du d abete Les Editio s Acta Med a Belg
2 HOET J J GOMMERS A & HOET J P (1958) 3rd I t Congress of the I tern ti n l Diabetes Dusseldorf July 24th
3 KRAHL, M E. (1953) J b l Chem 200 99
4 LIEBOW M I HELLERSTEIN H K & MILLER M (1955) *Amer J Med* 28 438
5 MACLEAN N & OGILVIE, R. F (1955) *D bet* 4 367
6 MADISON L. & UNGER R. H (1958) *J Cl In est* 37 631
7 VALLANCE-OWEN J (1958) 3 d Int Congress of the Inte national D betes Federation, Dusseldorf J ly 22nd
8 WILKERSON H L. C & ROMEIN (195) *Di betes* 6 324
9 WOOLF N & JACKSON W P U (1957) *J Path Bacteriol* 74 223

THE MEANING OF PRE-DIABETES IN PREGNANCY

J J Hoet A Gommers and J P Hoet

Laboratoire de Recherches de la Clinique Medicale
Hopital St Pierre Louvain Belgium

The appearance of the classical symptoms of diabetes mellitus – glycosuria with excessive thirst fasting hyperglycaemia craving for food weight loss acetonuria – does not indicate the beginning of the disease At that stage the damage of the pancreatic islets – and even other tissues – is usually irreversible

Tens of years prior to overt diabetes manifestations due to a relative and transient lack of insulin may appear during pregnancy

Our study has been focused on the disturbance of the maternal environment years before a pancreatic insufficiency i e overt diabetes mellitus results On the other hand the foetal anatomical structures and their development during intra-uterine life may be modified by the lack of insulin in the mother The impairments in the functions of the child s tissue may only appear later during extra-uterine life[1 2 6 10 1 13 15 17 19 4]

For many years clinicians have noted the high incidence in the obstetrical history of women over 40 years of age developing manifest diabetes of neonatal mortality stillbirths heavy babies and congenital malformations Basic research in this field shows that in female rabbits treated with sub-diabetogenic doses of alloxan pregnancy induced a loss of tolerance to dextrose Ricci[23] describes the increase in islet tissue and lipid infiltration during the last days of pregnancy in rats and also the very notable increase of capillary circulation of the islets during pregnancy *Post partum* this increased circulation returns to normal Many islets undergo lipid degeneration and disappear The number and volume of islets return to normal proportions in 2 or 3 days

Pregnancy in the woman is a test for islet function The transient insufficiency which may result and as a rule remains hidden or beneath a cloak is conditioned by the activity of the other ductless glands and in particular by the anterior lobe of

the pituitary the adrenal cortex and medulla the thyroid and the gonads The placenta as a source of progesterone and ACTH has also to be tal en into account[18 0–5]

These premises led to a more systematic study of carbohydrate metabolism during gestation The relationship between diabetic embryopathy and the changes in the maternal environment of the foetus become evident when the pregnant woman is more carefully studied regarding her *glycoregulation and metabolic disturbances*

Wilkerson and Romein[78] in research work undertaken in collaboration with the Children s Bureau United States Department of Health Education and Welfare make a comparative study o the outcome of mothers with and without symptomatic ... ot pre-diabetes in pregnancy These symptoms include a obstetrical history, large baby hyperglycaemia and diabetes in family This survey allows the conclusion to be drawn that t correction of the abnormal carbohydrate metabolism in diabetic mothers can bring about a reduction in the weight of th infants

A *study by Pyke*[2] *completing the* observations of Murphy Munro indicates clearly the diabetogenic role of repeated nancies Also a statistical survey by Beckers and Tuyns of the number of births in western Flanders (Belgium) in 1955 the decisive role of the number of pregnancies and of the age of mother in the development of large infants

I EXAMINATION OF MOTHERS OF CHILDREN WITH ... NORMALITIES

Downing and Goldberg[7] state that amongst 100 children ventricular septum defect 31 per cent had a family diabetes Amongst 100 children with inter-auricular d ... authors noticed 20 per cent of diabetic heredity by history There was only one mother who also had an inter-auricular Hence *the cardiac malformation* is seemingly not genetic in

After observing several cases of non-lethal congenital tion in children of diabetic or pre-diabetic mothers Hoet ... and Doyen clinically examined the *mothers of malformed* Twenty-six of the forty-eight mothers examined had features of an endocrinopathy especially diabetes hypoth ... or both

The fact that it is possible to show that an endocrine

present in the mother of a malformed infant, months or , the pregnancy leads to the suspicion that an endocrine d in pregnancy might play a decisive role in the genesis of formation.[11]

II. EXAMINATION OF PREGNANT WOMEN FOR CONCEALED DISTURBANCES

In order to observe the earliest phases of endocrinopathy nancy particularly pre-diabetes we examined those , women who had glycosuria in pregnancy or because of a pathology in pregnancy that gave reason for suspecting it of pre-diabetes Our observations concerned 500 mothers. histories were relevant for the birth of heavy infants, of m or still-born children, or for spontaneous miscarriage clinically recognizable cause[4,9,16]

An extensive familial and personal case history was taken complete physical examination performed with special emphas signs of endocrinopathy and of hypo- or avitaminosis

In all cases a Glucose Tolerance Test (G T T) was made as foll

1 In fasting state 100 g dextrose in about 200 c.c water

2 Capillary blood was taken and urinary collections made 0 hour ½ hour 1½ hours 2½ hours and 3 hours after dextrose ministration

3 Blood glucose was determined by the Hagedorn and J method The presence of true glucose in the urine was looked f with glucose oxidase paper (Clinistix or Testape)

4 The G T T was normal if Blood glucose

at 0 hr	½ hr	1½ hr	2½ hr	3 hr	
<1 g	<1 80 g	<1 30 g	<1 20 g	<1 00 g	‰

5 Repeated G T T were performed at short intervals in every woman with a history of perinatal mortality

A basal metabolic rate an examination of night vision by a Goldman-Weckers adaptometer showing haemeralopia and blood examinations for protein bound iodine liver function tests cholesterol and electrophoretic pattern of plasma proteins were performed A clinical study of 500 women showed their perinatal pathology (Table I)

A summary of the findings in relation to the glucose tolerance

TABLE I

PERINATAL PATHOLOGY OF 500 PATIENTS EXAMINED TO EXPLORE FOR CONCEALED ENDOCRINE DISTURBANCES

	Total
I *During gestation*	362
—with glycosuria primipara —45	
pluripara —71	116 (32%)
—miscarriage single (in primigr 20	
in plurigr 20	76 (21%)
repeated 36	
—with *macrosomia foetalis*	106 (29%)
—with children with congenital malformation	35 (9 7%)
—neonatal mortality (+ congenital malformation)	77 (21)
—diabetic heredity only	4 (1 1%)
—hydramnios hypertension oedema	14 (3 9%)
II *During post partum period*	101
because of	
—macrosomia foetalis	61 (60)
—neonatal death	28 (27%)
—child with congenital malformation	16 (16%)
—hydramnios hypertension oedema	2 (2%)
III *Non pregnant*	37
because of	
—miscarriage	27 (73%)
—congenital malformation	(5 4)
—sterility	2 (5 4%)
—macrosomia foetalis	7 (19%)
—stillborn	1 (2 7%)
—glycosuria	1 (7%)

curve during the pregnancy of 300 patients with perinatal pathology is given in Table II

TABLE II

GLUCOSE TOLERANCE TEST IN 300 PREGNANT PATIENTS WITH PERINATAL PATHOLOGY

Pathology	*No cases*	*Normal*	*Hyperglycaemia*			
			Flat	*Pre diabetic*	*Delayed*	*Pre-diabetic+delayed*
Glycosuria						
—primigravid	34	9 27%	6 17	5 15	14 41%	19 56
—plurigravid	55	17 37%	13 23%	7 12%	18 33%	25 46%
Miscarriages						
—in primigr	14	3 1%	4 29%	1 7	6 43%	7 50%
—single misc in plurigrav	15	4 6%	3 20%	2 13%	6 40%	8 53
—repeat misc	29	5 17	5 17%	8 28%	11 38%	19 66
Foetal macrosomia	91	26 8%	17 19%	9 10	39 43	48 53%
Stillbirths	59	15 25%	11 19%	8 14%	25 42%	33 56%
	297	79 26	59 20%	40 13	119 40%	159 53%

This survey shows that the perinatal pathology of selected cases is frequently associated with a disturbed carbohydrate metabolism in the pregnant woman The changes in the blood-sugar curve may be minor and have been classified under the heading delayed curves meaning that after 3 hours the fasting blood-sugar value (1 g ‰) was not reached. The blood-sugar values between 2 and 3 hours may be of importance in the evaluation of the disturbed carbohydrate metabolism of the patient In our selected cases this type of blood-sugar curve was found in 39 per cent of the cases during pregnancy

This type of data is important as Wilkerson[27] showed in his Oxford survey that 14 per cent of random people with delayed curves became overt diabetics in the same period This means that one has to be careful in interpreting the findings of blood-sugar curves of pregnant women

The pattern of the glucose curve during pregnancy is very variable A normal blood-sugar curve at the beginning of the pregnancy will not necessarily remain normal throughout the pregnancy A pregnant woman with a delayed curve at the beginning of pregnancy may have a pre-diabetic or a diabetic curve at the end None of the pregnant women included in this study had a fasting blood sugar above 1 10 g ‰ Occasionally a pre-diabetic curve of an insulin treated woman will become normal or even flat during or after the pregnancy

It has to be stressed also that the glucose tolerance test has to be analysed with respect to heredity the perinatal pathology and the time of the observation in regard to the pathological events The metabolic disturbances will be concealed especially in cases of congenital malformation or stillbirth as a major endocrine disturbance will not allow a person to become pregnant or to remain pregnant The characteristic of the pathological picture is that the mother does not complain of any specific symptom her biological disturbance however is severe enough to affect the developing foetus

Three clinical histories will illustrate (1) the changing pattern of the glucose tolerance test depending upon the time of observation (2) the beneficial effects of adequate insulin therapy (3) the association of pre-diabetes with other endocrine disturbances which may be as important as pre-diabetes itself

Case 1 *V L* (294) 27 years old – consulted physician for the first time 2 months after her third pregnancy

In 1952 she delivered a stillborn baby with spina bifida premature at 8 months weighing 3 kg 250 g In 1953 her second pregnancy terminated in a stillbirth The foetus weighed 3 kg 250 g —it had a spina bifida In 1954 a third infant was born alive affected with a meningomyelocoele hydrocephalus and clubfeet weighing 4 kg 200 g dying at 6½ months

Family Case History Maternal grandmother diabetic (six stillbirths)

Personal Case History The patient weighed 4 kg 200 g at birth She had *complained of polydipsia polyuria and of sensitivity to cold during* her pregnancies She had frequent conjunctivitis and blepharitis In 1954 2 months after the last of her three pathological pregnancies her G T T was as follows with 50 g of dextrose

1 03	1 5-	1 61	1 36	0 93	
(—)	(—)	(—)	(—)	(—)	(without glycosuria)

Her physical examination showed marked keratoxanthosis of the sole of the feet She had webbed toes on the right foot

In 1955 the patient was again pregnant and complained about polydipsia and pruritus vulvae *her G T T at 6 weeks pregnancy was*

0 91	1 78	1 52	
(+)	(+)	(+)	(glycosuria)

She was treated with 10 to -8 units of N P H insulin and 50 000 units of vitamin A daily Shortly before confinement delivery was induced The patient delivered a healthy boy of 3 kg 550 g Ten days after her delivery her G T T was

0 84	1 91	1 93	1 84	1 37	
(—)	(+)	(+)	(+)	(+)	(glycosuria)

One month later her G T T was

0 81	1 59	1 31	1 05	0 90	
(—)	(—)	(+)	(tr)	(—)	(glycosuria)

In 1957 the patient was again pregnant At 4 weeks pregnancy she had a G T T as follows

0 88	2 08	1 71	
(—)	(+)	(+)	(glycosuria)

Her dark adaptation was 1 2 lower than normal (1 6) She received 20 units of N P H insulin at first and this was increased up to 36 units N P H. in the morning and *16 units crystalline zinc insulin in the evening* She was delivered again of a healthy daughter weighing 3 kg 440 g

Case 2 *G R* 30 years old – consulted physician during her third pregnancy because

In 1951 she was delivered of her *first* baby with congenital cardiac malformation The boy weighed 3 kg 900 g In 1953 she was delivered

a second time of a girl with cardiac and renal malformation, weighing 3 kg 600 g It died - days later

The patient s birth weight was 4 kg When presenting in 1954 she was in the second month of her third pregnancy Her G T T was as follows

0 95 1 91 1 48 1 33

She was treated with -0 units globin zinc insulin At 3 months the G T T was

1 31 1 95 1 59 1 34

She was delivered spontaneously 2 weeks prematurely of a healthy girl of 3 kg 400 g Six weeks after the delivery the G T T was

0 82 1 78 1 47 1 -1

In 1956 she was again pregnant and was treated again with 20 to 24 units globin zinc insulin At 6 months her blood sugar curve was as follows

0 94 1 69 1 64

She was delivered spontaneously of a healthy boy weighing 3 kg 700 g Six months after the delivery the G T T showed

0 82 1 79 1 50 1 19

She was feeling well and did not receive any insulin then

Case 3 S M 29 years old — was first seen 5 days after the delivery of an 8½ months premature stillbirth

Past Case History is relevant for a thyroidectomy for goitre in 1950 The type of goitre remains in doubt

Perinatal Case History In 195- she was delivered of a healthy girl weighing 3 1 kg During pregnancy she complained of oedema and occasional vaginal bleeding Hypertension and albuminuria had been found. In 1958 she was delivered after 8½ months of a stillborn baby Five days later a G T T was as follows

0 66	1 7-	1 77	1 81	0 63	
(—)	(—)	(—)	(—)	(—)	(without glycosuria)

Four weeks later

0 80	1 60	0 93	0 87	0 76	
(—)	(—)	(—)	(—)	(—)	(without glycosuria)

B Met —8 per cent

She was being treated with 100 mg thyroid extract This case history is relevant for a thyroidectomy and a history of stillbirth Five days *post partum* the blood-sugar curve was definitely delayed Four weeks *post partum* the glucose tolerance curve became normal The patient had hypothyroidism with an associated pre-diabetic tendency during pregnancy

The close observation of many cases of pregnant hypothyroid women brought the opportunity to follow the progressive deterioration of the G T T as a result of thyroid therapy during pregnancy

Therefore we consider that when thyroid extract is prescribed and the G T T becomes pre-diabetic insulin has to be given to avoid more profound carbohydrate disturbances The outcome of five cases of associated therapy with thyroid and insulin are reported (Table VI)

This association is seen even more frequently in recent cases

These three examples illustrate the fact that pregnancy induces quite frequently a diminished tolerance to glucose The pregnant woman has no specific complaints or does not show any symptom This disturbance in carbohydrate metabolism may still affect quite adversely the foetal development

This has been investigated in fifty patients who were previously delivered of children with congenital malformation Sixteen of the patients were seen in the *post-partum* period of the birth of the affected child The glucose tolerance tests of these sixteen women are reported in detail

The glucose tolerance curves in the *post-partum* period may not show evident pathological changes and will not preclude abnormal carbohydrate metabolism during pregnancy Therefore the glucose tolerance tests in thirty-four mothers of malformed children were analysed during their next pregnancy The detailed data are tabulated in Appendices A B C D

Among thirty-four mothers ten had a normal G T T seven a flat G T T eight a delayed G T T and nine a pre-diabetic G T T The patients with a flat glucose tolerance test had strikingly a rather low basal metabolism for the period of pregnancy

TABLE III

GLUCOSE TOLERANCE TEST IN FIFTY MOTHERS OF CHILDREN WITH CONGENITAL MALFORMATION

		Glucose tolerance te t							
	Total	Normal		Fl t		D layed		Pre-d abetic	
		Total	p r cent	Tot l	per cent	Total	per cent	Tot l	per cent
Post partum	16	6	38	3	19	5	31	2	12
Pregnant	34	10	29	7	21	8	24	9	6

The data indicate that the birth of a child with a malformation may be associated with a disturbed glucose tolerance in the mother during pregnancy This disturbance might be *transitory* and might only be present during pregnancy

The data show that only 12 per cent of these mothers had a pre-diabetic curve during their *post-partum* period whereas 26 per cent had a pre-diabetic curve during pregnancy (Table III and Appendix E)

A similar observation has been made in women having case histories of stillbirth (Table IV) The pathological disturbances of carbohydrate metabolism are striking among thirty two women eighteen had an abnormal glucose tolerance curve immediately after the stillbirth During a new pregnancy about the same incidence of disturbances were found (Table IV)

Like the woman delivering a child with a congenital malformation a woman with a case history of stillbirth or miscarriage is very likely to have an abnormal glucose tolerance curve during a next pregnancy – about 40 to 50 per cent of the women with perinatal wastage may show among other endocrinopathies a disturbed glucose tolerance curve when pregnant

The following data indicate that the correction of the endocrinopathy has a beneficial effect on the outcome of pregnancy The

TABLE IV

GLUCOSE TOLERANCE TEST IN EIGHTY-FIVE WOMEN WITH A HISTORY OF STILLBIRTH

	Total	*Normal*	*Flat*	*Delayed*	*Pre-diabetic*
During new pregnancy					
A. Preceding pregnancy ended in stillbirth	27	9 33	8 30	8 30	2 7
B One of the previous pregnancies ended in stillbirth	26	10 38	2 8	11 42	3 12
During post-partum (after stillbirth)	32	5 16	9 28	9 28	9 28

TABLE V

GLUCOSE TOLERANCE CURVES IN EIGHTY THREE PREGNANT WOMEN WITH A HISTORY OF MISCARRIAGE

	Total	*Normal*	*Flat*	*Delayed*	*Pre-diabetic*
Miscarriage/gravida I	22	4 18	6 27	10 45	2 10
Miscarriage/mult gravid	22	6 27	3 14	10 45	3 14
Repeated miscarriages	39	7 18	6 15	17 44	9 23
Total	83	17 20	15 18	37 45	14 1

aetiological treatment of the metabolic disturbance influences favourably the foetal development

The observations concern the outcome of pregnancies when mothers with perinatal wastage have been treated with the maximum amount of insulin with thyroid extract (50–200 mg /day) if indicated and with vitamin A (20 000–50 000 units/day)

Pregnant women having delivered previously a child with malformation 34
Pregnancies of the treated women before treatment 69
Pathological pregnancies of the treated women before treatment 35
Successful pregnancies after aetiological treatment 18
Pathological pregnancies after aetiological treatment 0

Hence the untreated pre-diabetic mother as well as the poorly treated diabetic mother is liable to have children with congenital malformation The aetiological therapy diminishes greatly the risk of such an outcome

The beneficial effects of endocrine-substitution therapy on the prevention of perinatal wastage are also quite obvious from the results obtained by the aetiological treatment in cases of concealed endocrinopathy with other manifestations of perinatal wastage (e g stillbirths miscarriages)

Table VI summarizes these results on the total number of pregnancies after the aetiological treatment of the metabolic disturbance of the mother

TABLE VI

RESULTS OF THE TREATMENT OF CONCEALED ENDOCRINOPATHY ON PERINATAL WASTAGE

	Pre-diabetic pregnancies		*Hypothyroid pregnancies*		*Pre-diabetic hypothyroid pregnancies*	
	Before insulin R	*With insulin R*	*Before thyroid R*	*With thyroid R*	*Before* thyroid + insulin R	*With* thyroid + insulin R
Number of pregnancies	95	35	38	14	32	5
Per cent of normal pregnancies	0 /	77 /	19	70	13	80
Per cent of pathological pregnancies	48	3	72 /	7 /	74	20 / *
Per cent of foetal macrosomia	32 /	20	9	3	13 /	0

* The single pregnancy terminated in a stillbirth—Rh incompatibility was associated with concealed endocrinopathy A high titre of anti-Rh bodies was present in the mother's serum

III THE EFFECT OF INSULIN ON DEFECTIVE DARK ADAPTATION AND CAROTINODERMIA

Relationship betu een Disturbances of Carotene Metabolism during Pregnancy and Foetal Pathology

(In collaboration with Dr F Stein)

A factor common to the pre-diabetic and hypothyroid pregnant woman is carotinodermia and defective dark adaptation The latter is measured by the adaptometer of Goldman-Weckers In a pre-diabetic patient the defective dark adaptation will improve by insulin treatment The following cases illustrate this statement

Case 1 L S (Fig 1*a*) A 29-year-old married male father of one consulted his physician because of increasing fatigue and somnolence over the last few months His father was a known diabetic for the last 5 years of his life and he had been treated with insulin

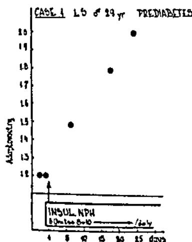


Fig 1a

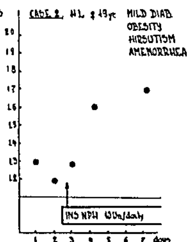


Fig 1b

Patient's physician found glycosuria and referred the patient to the diabetic clinic for a glucose tolerance curve (100 g dextrose with a blood sugar every 45 minutes) 0 83 — 1 33 — 1..9 — 1..1 — 1 07 without sugar or acetone in the urine The glucose tolerance test was not pathological

notwithstanding the blood-sugar did not reach fasting levels within 3 hours after glucose intake

The adaptometry* (measurement of night blindness) was low 1 . at two occasions after 7 days of treatment with 6 units of insulin NPH the adaptometry rose to 1 5 and 10 days later with 8 units of insulin NPH to a normal value of 1 8 Twenty-three days after insulin treatment the adaptometry was 2 0

Case 2 H L (Fig 1*b*) A 19-year-old girl was referred to hospital because of obesity hirsutism and amenorrhoea She weighed 87 6 kg height 1 57 m No major metabolic abnormality could be found but a pathological glucose tolerance curve (100 g glucose with a blood sugar every 45 minutes) 1 03 g ‰ — 2 49 — . 13 — 1 65 — 1 30 with sugar in the urine

The adaptometry was 1 3 repeat 1 2 before insulin treatment 24 hours after insulin treatment adaptometry was still 1 3 3 days after treatment it was 1 6 and 5 days after 10 units NPH daily it was 1 7

Case 3 A V (Fig 2) A 32-year-old mother of two is referred to the hospital because of myxedema She had been treated for diagnosed hyperthyroidism with radioactive iodine 6 years ago

At the time of entry her subjective symptoms and physical examination were classical for myxedema She was characteristic for marked xanthodermia and paleness Her adaptometry before treatment was 0 9 She was then treated with tri-iodothyronine in increasing dosage 25 γ 50 γ 75 γ a day Her adaptometry rose quickly up to 1 2 After 1 month on 75 γ tri-iodothyronine a day the adaptometry was 1 6 The patient was then started on thyroid 50 mg a day Her adaptometry was still 1 6 after 2 weeks of thyroid

We consider that this improvement of the vitamin-A genesis at the level of an effector organ the retina was an indication of some of the events at the level of the foeto-placental tissues

Hale and Warkany Wilson and Schappenberg described hypovitaminosis A as a teratogenic condition We consider that prediabetes and hypothyroidism produce an insufficiency of vitamin A at the level of the effector organ with the greatest need [4 9 14 6]

Recently Millen and Woollan[18] have seen that experimentally hypervitaminosis A has a teratogenic action in the rat This teratogenic effect was definitely potentiated when cortisone was injected during

* The mea urement of dark adaptation (ad ptometry) is expressed as the logarithm of the light intensity in lux, that one is able to detect in 25 after a prolonged expo ure to bright light Normal values are above 1 6 The values under 1 6 are bnormal and they show a poor cap city of d rk adaptation

pregnancy In complementary experiments they demonstrated a close insulin-cortisone relationship in experimental teratogenesis The experiments showed that insulin prevents wholly the potentiating action of cortisone on the teratogenic effects of hypervitaminosis A

Experimental hypothyroidism during pregnancy as the result of administration of 4 methyl 2 thiouracil also has a potentiating effect on the teratogenic activity of hypervitaminosis A

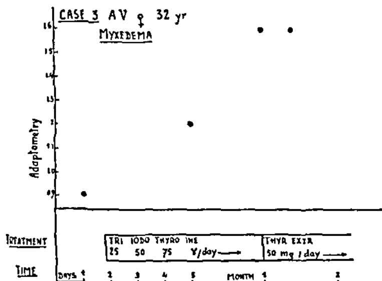


Fig. 2

Therefore we underline the normalization of the dark adaptation as the result of insulin treatment The improvement of the nutrition of the placental and foetal tissues was shown by the outcome of the pregnancies in those women with a long record of pregnancy wastage

These facts point to an action of insulin and thyroid extract on a metabolic pattern involving carotene and its dynamic equilibrium with biologically active vitamin A The gross and permanent disturbance of glucose tolerance test is a later symptom of the progressing disease

The link between pre-diabetes in pregnancy and the foetal pathology and the congenital malformations is not a direct one Some more specific damaging factors other than hyperglycaemia may cause the embryopathy The example of the disturbance of the vitamin-A metabolism is probably not the only one[14]

The maternal environment of a concealed pre-diabetic mother may be unfavourable for the foetus through more than one factor The moment and degree of the pathological metabolic disturbance will determine the nature of the events Early in pregnancy it may be a miscarriage later a stillbirth with gross anomalies in milder cases a liveborn baby may just show an adaptation crisis to extra-uterine life In other cases a live newborn may have a pancreas with numerous hyperplastic islets (Dubreuil *et al*[35]) The overweight newborn with a Cushing aspect is an easy retrospective fact in the clinical history[8] The anatomical lesion of the foetal pancreas is a sign of embryopathy rather than the exclusive result of genopathy

Careful studies may produce an explanation of the phenomenon of anticipation of diabetes[-5] The natural history of infantile and juvenile diabetes must be considered in the light of possible intra-uterine pancreatic changes A foetus carrying a diabetic gene may be further damaged by a disturbed maternal environment For instance the offspring of the diabetic father may be submitted to an unfavourable maternal environment of a concealed pre-diabetic mother

On the other hand non-lethal congenital malformations in the offspring of diabetic parents or the association of all varieties of anomalies with clinically more or less severe diabetes mellitus points to embryopathy We focus the attention on observations of syndactylus situs inversus renal malformation haemangiomata bifid uterus mongolism Klippel Feil syndrome and supernumerary nipples associated with diabetes mellitus

Conclusions

From a practical point of view the glycosuria and the G T T *during pregnancy may not* be isolated from the other clinical data To detect concealed metabolic disturbances which may have considerable effects on the foetus it is of importance

1 To acquire a maximum of gravido-puerperal data of the

pregnant mother s relatives in particular birth weight stillbirth or congenital anomalies of sibs

2 To look for minor clinical signs expressing metabolic disturbances like xanthosis keratoderma cheilosis glossitis anaemia acetonuria night blindness emesis pruritus vulvae excessive weight gain or loss

3 To establish the association of pre-diabetes with other endocrine pathology hypothyroidism is frequently associated with pre-diabetes (importance of goitre) Virilism may point to adrenal disturbances

4 To repeat G T T if perinatal pathology has been severe (stillbirth) Protein bound iodine electrophoresis and basal metabolism will be checked regularly

5 To be alert about endocrinopathies in the parents which may point to possible foetal pathology

Summary

Pre-diabetes during pregnancy has been discovered in a clinical survey of mothers of malformed children or with pregnancy wastage syndromes

Covering 500 cases of women selected on these grounds it may be stated that 50 per cent present disturbances of the G T T

These symptoms show up by predilection during pregnancy Minor alterations before pregnancy become frankly pathologic during gestation

Hypothyroidism may be associated with a disturbance of carbohydrate metabolism.

These studies gave the opportunity to treat eighteen pregnant women affected with endocrinopathies

The pregnancy wastage for the previous gestations reached thirty-five out of sixty nine

Eighteen pregnancies carefully treated and controlled gave live newborn without a single loss

Attention is focused on the intimate relationship of pre-diabetes or hypothyroidism with clinical signs of hypo-vitaminosis A This might be one of the metabolic factors of the teratogenic action of the minor endocrinopathies of pregnant mothers Incomplete treatment of overt diabetic women results in the same foetal pathology

We acknowledge thankfully the collaboration of Professor J A Schockaert Professor M Renaer Drs Osinski Pagni Wattieg Mr Ekka and of the assistants of the Lying-In and of the Cliniques Universitaires St Pierre Louvain

This work has been performed with the technical assistance of Messrs L Rutten G Crombez and Miss F Hottelet

This survey has been sponsored by the Fondation Reine Elisabeth the Josiah Macy Foundation N Y U S A, and the Fonds National pour la Recherche Scientifique

REFERENCES

1 BASTENIE P (1956) Corticosurrénale et diabète humain *Ed Masson* p 271
2 VAN BEEK, C C (1939) *Ned T voor Geneesk* 83 5973
3 VAN BEEK C C (1952) International Congress of the International Diabetes Federation Leyden Holland
4 BERGQUIST N (1954) *Acta endocrinol* 15 166
5 CARDELL B S (1953) *J Path & Bact* 66 335
6 CARRINGTON E R *et al* (1957) *Obstetrics & Gynecology* 9 664
7 DOWNING D F & GOLDBERG G (1956) *Disease of the Chest* 29 475
8 FOCKEN A K (1954) *Ze tschr Klin Med* 151 199
9 GIROUD A (1954) *B ol Rev* 29 220
10 HOET J P (1954) *D abetes* 3 1
11 HOET J P BRASSEUR L & DE MEYER R (1955) *Ann d Endocrinol* 16 17
12 JACKSON W P U (1952) *Brit med J* ii 690
13 JACKSON W P U & WOOLF N (1957) *La cet* i p 614
14 JONES G M GUEST G M & JACKSON R L *et al* (1956) *Diabetes* 5 303
15 KATSCH G (1950) *Zentralbl f Gynaekol* 24 1956
16 MAURIAC P (1952) Retouches au tableau cl ssique du diabète *Ed Masson* 1
17 MILLER H C (1946) *J Ped trics* 29 455
18 MILLEN J W & WOLLAM D (1958) *Nature* 181 418
19 MOSS J M & MULHOLLAND H B (1951) *Ann Int Med c ne* 34 678
20 MURPHY R (1957) *Connect cut M J* 21 306
1 PEDERSEN J (1954) *Diabetes* 3 199
2 PYKE, D A (1956) *Lancet* 1 818
23 RICCI, P D (1958) *Folia Endocr nolog ca* 11 5 1
24 SINDRAM I S (1955) *Thèse* Amsterdam 1 313
25 STEINBERG A G (1955) *Diabetes* 4 126
6 WARKANY J *et al* (1956) *Ped atrics* 19 719
27 WILKERSON H L C & KRALL, L P (1953) *J A M A* 152 1322
28 WILKERSON H L C & ROMEIN G R (1957) *Diabetes* 6 324

APPENDIX A

GLUCOSE TOLERANCE TEST DURING PREGNANCY OF THIRTY-FOUR WOMEN HAVING DELIVERED PREVIOUSLY A CHILD WITH CONGENITAL MALFORMATION

A. Normal Curves 10

N	Anamnesis: Malformation	Anamnesis: Birth weight kg	Anamnesis: G da	Present pregnancy: Gravid	Present pregnancy: Month preg	Glucose tolerance test gm %: 0	½ h	1½ h	2½ h	3 h	B.M.R.	Vt A γ	Adipt NI 1 6
100	Polycystic kidney	4 000	1st	3rd	8th	0 87	1.42	1 07	1 18	0 61	+27		
81	Congenit. l x. h p	2 200											
	Little d sease	8 m	1st	2nd	nd	0 76	1 19	1 00	0 91	0 89			1 8
103	Card. malform.	3 000	1st	3rd	4th	0 75	20	1 27	1 04	0 66			
03	Card malform	3 300	1st	2nd	7th	0 88	1 34	1 2	1 09	1 21			
23	Spina bifida												
	Men goroele	2 850	1st	2nd	3 d	0 67	1 45	1 29	1 15	0 73	+26	37	0 8
430	?	3 000	9th	11th	6th	0 73	1 35	1 28	1 4	0 82	+27	105	1 9
305	Anenceph 1	2 7 m	1st	3rd	d	0 76	1 25	1 10	1 02	0 8			
465	Unknown malf	1 700		2nd	4th	0 86 I V	2 15	0 50	0 51	0 74	−23		
476	Twins												
	Cardiac malform	2 600	1st	nd	3 d	79	1 37	09	1 00	1 7			
	Oesoph. stenos	1 900											
4 8	Spina bifid	3 000	nd										
	Spi a b fida	3 650	3rd										
	Spin l fida	3 650	4th	5th	4th	0 83	51	1 19	1 10	1 07	+12		

Appendix B

GLUCOSE TOLERANCE TEST DURING PREGNANCY OF THIRTY FOUR WOMEN HAVING DELIVERED PREVIOUSLY A CHILD WITH CONGENITAL MALFORMATION

B Flat curves 7

Anamnesis			Present pregnancy									
					Gluco toler nce test gm %							
n	B rth w ght kg	Gravid	G avi da	Mo th pregn	0	½ hr	1½ hr	2½ h	3 hr	B M R	I t A y°	Ad pt Nl 1 6
2 +												
2 E	2 150											
encephalus	2 8 m				0 93	1 22	0 73	0 63	0 73	—8		
sence of femur	3 200	2nd	2nd	3rd	0 93	0 89	0 87	0 62	0 60	—7		
nosis of oesphagus	3 000	1st	3rd	4th	0 93	1 24	0 95	0 99	0 63	+2		1 4
nt malf	3 500	3rd										
idneys	4.050	4th	5th	4th	0 73	1 04	0 95	1 08	0 95	—8		
us	3 500	1st										
is	?	2nd		3rd	0 68	1 13	0 97	1 06	0 81	+2		
ys	3 100	1st										
ys	3 550	2nd										
seys	3 700	3rd	5th	2nd	0 89	1 26	1 16					
e malf.	2 800	1st										
	at 8½ m		ad	3rd								

APPENDIX C

GLUCOSE TOLERANCE TEST DURING PREGNANCY OF THIRTY-FOUR WOMEN HAVING PREVIOUSLY DELIVERED A CHILD WITH CONGENITAL MALFORMATION

C Delayed curves 8

	Anamnesis			Present pregnancy									
						Glucose tolerance test gm ‰							
No	Malformation	Birth weight kg	Child	Gravida	Month pregn	0	½ hr	1½ h	2½ hr	3 h	B.M.R.	I.F.A. γ ‰	Ad. NI 1 6
306	Spina bifida	4 200	1st	2nd	4th	0 77	1 55	1 41	1 25	0 92	+12	64	
372	Spina bifid m lf k doeyt	3 100	4th	5th	4th	0 78	1 0	1 27	1 39	0 92			
412	Spina bifida	2 900		4th	6th	0 74	1 75	1 72	1 08	0 7			
197	Meningocoel	4 150	1st	4th	8th	0 95	58	1 48	1 25	0 68	+48	37	
433	Spast p ral	3 10	1st										
	Sp. paral	3 130	3rd	4th	1st	0 79	1 18	1 39	1 49	0 81	+8		2
173	Spina bifida	2 500	4th			0 85	1 60	1 38	1 33	1 01	+13		
517	Hydrocephalus	2 800	3rd										
	+ ocul. malf	1 8 m.		4th	4th	0 82	1 64	1 64	30	32	+4		1 3
519	Sp a bifida		6th										
	Hydrocephalus			7th	5th	0 82	1 46	1 33	1 68	1 23	+10		

APPENDIX D

GLUCOSE TOLERANCE TEST DURING PREGNANCY OF THIRTY-FOUR WOMEN HAVING DELIVERED PREVIOUSLY A CHILD WITH CONGENITAL MALFORMATION

D Pre-diabetic curves 9

No	At amnes s				Present preg sancy									
						Gl cose tolerance test gm /oo								
	M lformat n	B rth we ght kg	G a t da	C avs da	M nth pregn	0	½ hr	1½ h r	2½ h r	3 h r	B.M R.	V t A γ	Adapt Nl 1 6
334	Sp na bifida	3 600	3rd	4th	5th	0 90	1 77	2 35	1 70	1 12			
274		2 200										64	
		at 8 m.	1st	nd	3rd	1 21	2 41	2 47					
372	Card malform	4 000	4th	5th	6th s	1 01	1 67	2 12	1 97	2 07	—1		1 9
314	Anencephalus	3 00	2nd		3rd	0 84	1 84	1 57	1 04	1 98	+7		1 7
324	Oesoph. stenosis	2 300	2nd	3rd	4th	0 97	2 41	1 51			+17		2 7
13	Spina b f da	2 500	4th		5th	0.86	1 94	1 58	1 12	0 79	+27		
31	Anencephalus	? at 6 m.	1st		2nd	0 90	1 83	1 70	1 68	0 97	+16		2
94	Spina bifida	3 250	1st										1 7
	Sp na bifida	3 250	2nd										
	Hydroceph												
	Club f et												
	Meningomyeloc	4.200	3rd										
75	Card. malform	3 900	1st			1 00							
	Ca d. + renal malform.	3 2											

APPENDIX E

GLUCOSE TOLERANCE TEST DURING THE 'POST-PARTUM' PERIOD OF SIXTEEN PATIENTS AFTER THE DELIVERY OF A CHILD WITH CONGENITAL MALFORMATION

N	Malformation	Birth weight kg	Gravida	Age mother	Glucose tolerance test gm. ‰ 0	½ hr	1½ h	2½ hr	3 hr	Vit. A γ	Ad pt NI 1.6
A Normal G T T 6											
132	Hydrocephalus Spina bifida		1st	26	0.86	1.35	1.26	0.93	1.05	57	
184	Spina bifida Club feet	3.450	1st	23	0.69	1.26	0.98	0.81	?	18	
440	Cyclops Malf. thorax	2.200	2nd	24	0.83	1.49	1.08	0.87	0.76		
438	Anencephalus	3.000	2nd	24	0.92	1.27	1.09	1.01	1.08		1.3
34	Sex undiffer Club feet Harelip	2.330	3rd	27	0.74	1.29	1.20	1.18	1.03		
149	Malf. card.	3.700	1st	24	0.83	1.76	1.15	1.08	0.65	64	
B Flat G T T 3											
410	Harelip	4.480	2nd	33	0.85	1.04	0.79	0.83	0.56		
469	Cleft palate	3.200	2nd	25	0.93	0.99	0.92	0.70	0.67		
505	Meningocele	?	2nd	29	0.86	1.13	0.97	0.95	[illegible]		0.9
C Delayed G T T 5											
37	Hydrocephalus		1st	25	0.92	0.94	1.79	1.12	1.41		
41	Meningocele	3.250	1st	24	0.63	[illegible]	1.52	1.30	1.18		
379	Spina bifida	?	1st	25	[illegible]	1.49	1.1	1			
482	?	1.7 m	1st	36	0.86	1.62					
83	Anencephalus	2.750	4th	24	0.81						
D Pre-diabetic G T T 2											
30	Harelip										

DISCUSSION

TUNBRIDGE Professor Young in opening the meeting suggested that we should let our hair down and thus I shall proceed to do as far as nature permits! After the excellent presentations by Dr Jackson and Professor Hoet a clinician may be permitted to ask a series of questions (1) What do we understand by the term diabetes mellitus? (2) What do we mean by control? (3) What are the causes of the so-called complications of diabetes mellitus? And lastly (4) How may they be prevented, since they provide the major problem for the clinician treating diabetes mellitus today?

Diabetes mellitus is really an umbrella term used to cover all cases exhibiting hyperglycaemia A raised blood-sugar level is the lowest common denominator for the making of the diagnosis Despite numerous attempts to classify diabetes either according to insulin dosage or insulin resistance or sensitivity the age of the patient or according to somatotype no satisfactory clinical classification has yet been devised

The problem as Dr Jackson so clearly indicated, is whether an abnormal glucose tolerance test indicates pre-diabetes or diabetes mellitus The majority of clinicians will accept that a patient presenting with a history of sepsis and ketosis *even if at a subsequent date they* had a normal glucose tolerance test should be considered either as a pre diabetic or as a potential case of diabetes mellitus The interpretation of minor differences in the glucose tolerance test is often a matter of opinion and therefore conclusions as to the significance of these changes are very difficult to interpret The follow-up of cases showing minor degrees of abnormality in a glucose tolerance test over a period of at least 10 years have been very few and such reviews as have been carried out would indicate that rather more than half of such patients ultimately develop diabetes mellitus but by no means all of them The problem for the clinician, is how to make an accurate prognosis in the absence of any family history of diabetes The recently introduced cortisone test may assist in the differentiation but it is too early yet to say how reliable this will prove To revert to the patient who has had an *initial incident of* glycosuria and after treatment had apparently maintained normal health for 20 years but who then presents with severe arterial degeneration and retinopathy *Has he had active phases of diabetes over the 20 years* which have been undiagnosed or has he had a silent hyperglycaemia for 20 years? In other words is hyperglycaemia the only criterion of diabetes *mellitus or are there others which at the* present time we have no means of assessing and are therefore overlooked? This raises a most important problem and one which is at the root of any policy that we must adopt concerning both the diagnosis and treatment of diabetes mellitus When we come to consider the problem of the control of diabetes mellitus we

are on difficult ground because we do not know what we are There are excellent reports of short term control in hospital c bolic wards but there is very little satisfactory work on the trial In Sweden they carried out a survey by questionnaire n opinion a very satisfactory method. They wrote to 5,07 patients and asked them whether they had followed their diabet Thirty-six per cent indicated that they had followed the diet well, 54 per cent admitted they had not followed it very caref 10 per cent not at all I tried a simple experiment in my own cl random sample of those living in the city of Leeds largely becau convenience of visiting The random sample numbered one hundr five and ninety four of these patients co-operated. In sixteen d followed exactly as one should expect in thirty-six the diet was satisfactory but in the remainder hopeless In Arrhus Lundbeck went into the problem and analysed very carefully all the factors cerned. I think we can say that the most important of all is that of direct personal supervision We should assume that at best only 15 per cent of our patients however well we teach them and how frequently we see them will really adhere to their diet over a long peri of time Another problem in assessing the effect of diet is the fact th fashions change and over the last 40 years there have been conside changes in the approach to the dietary control of diabetes

What is often not realized are the wide changes which may take pla in the blood-sugar level during the 24 hours even in a patient who appea to be satisfactorily controlled. My colleagues and I have already drawn attention to this in assessing the insulin requirements and it is not at all uncommon for a sensitive diabetic to have short periods of ketosis lasting for a quarter or half an hour associated with a blood sugar of 200-250 mg per cent and yet during the remainder of the 24 hours have a blood-sugar level below 200 mg per cent It is these facts which make using the blood-sugar level the principal indication of adequate controls so difficult

This leads to my third point the question of the relationship of the so-called complications of diabetes mellitus to the control of the hyper glycaemia. Dolger in 1947 first hinted that the control of the hypergly caemia did not make any difference to the development of complications The Boston school have always stressed the importance of good control and have published many papers suggesting that good control was followed by a lowered incidence of complications Lundbeck, in his very careful follow-up of children in Arrhus went no further than to say that the evidence suggested that retinopathy which is really the only objective complication we can diagnose early was significantly less in those whom they assessed as having been well controlled.

In discussing the whole problem of complications I think it is necessary

to divide them into two groups the retinopathy neuropathy and nephropathy which seem to be specific for diabetes and the widespread vascular degeneration which histologically is not different from that found in old age The only possible difference is that in diabetes it is found very widespread and in veins as well as arteries The specific nature of the first group is perhaps illustrated by the following case the patient was first seen at age 30 when she had had diabetes for only 5 days She was a school teacher and very co-operative and during the 4 years that I attended her she was extremely careful with her diet and never to my knowledge had a blood sugar over 200 mg per cent until just before her death Retinal haemorrhages were first observed 3 years after the onset and she died 18 months later having developed severe retinitis proliferans and severe renal lesions The post mortem examination revealed extensive Kimmelstiel-Wilson changes but only slight evidence of atherosclerosis This patient seemed to illustrate the specific nature of certain vascular changes found in association with diabetes mellitus and to emphasize the point I have already made that there are other disorders in diabetes mellitus besides hyperglycaemia for which at the present time we have no reliable method of assessment

On the other hand in the elderly diabetic one does find a very high incidence of coronary disease and arterial degeneration In other words it seems as though senescent changes were occurring a decade earlier than in normal people In a series of nearly 400 autopsies in which I compared the gross findings in elderly arteriosclerotics in people who had had glycosuria discovered for the first time just before death and in those who were known to have had diabetes for many years there was no gross difference in the incidence of atheroma in the major vessels of the heart or the brain

This brings me to my final point namely that of prevention The acceptance of the views of Dr Jackson and Professor Hoet would be valuable because it is conceivable that if we could control hyperglycaemia we might lessen the present-day clinical picture in the treated diabetic and possibly lessen the high incidence of complications On the other hand there is considerable clinical evidence that hyperglycaemia is by no means the whole story and we should be on our guard against accepting hyperglycaemia as the only metabolic abnormality in diabetes mellitus

Before closing I would like to pay a tribute to our hosts It has been very stimulating to clinicians to hear of the experimental work being done in relation to diabetes mellitus and naturally we may find it difficult to apply immediately the information we have gained Professor Young has said that we have been possibly a little removed from the treatment of patients but I think that he and all of us may rest assured that we have been dealing with a very important problem because in Great Britain

alone within the next 25 years nearly 1 per cent of the pop likely to develop diabetes mellitus although we shall probably made the diagnosis in about half the cases

OAKLEY I just want to make two points both to emphasize h I agree with Professor Tunbridge about two points he made on the subject of the classification of diabetes into clinical types this has been very useful but that it can be overdone I think th very hard and fast classification which could be regarded as sati and that any grouping of diabetics leaves a great deal of interme who cannot conveniently be fitted into any one particular gro mention this because I think we are apt to forget what I regard as a important observation which was described I think by Dr Jones — sorry I am not quite sure of his name He described a group of ca the West Indies in which he had these young diabetic patients who highly insulin resistant who showed not the slightest tendency to k and were a complete reversal of the accepted picture in Europe Follow that recently I have had a report from Ceylon of a series of cases hibiting exactly the same manifestations and it is rather significant the two groups have very much the same and rather peculiar and uniq feeding habits I think this tendency to force diabetics into certain gories is liable to make one overlook those groups which do not fall i any such category and such groups I think will very greatly reward n work and study

The other point I wanted to make was regarding arterial complications I was delighted to hear Professor Tunbridge say what I myself have found in a follow up of 2460 patients in my clinic in which we studied the incidence of peripheral arterial disease and we found no correlation whatever between the duration of the disease the severity of the disease and the presence or absence of peripheral arterial lesions We did, how ever find a significant and consistent relationship between such disease and the age of the patient I would agree entirely with him that we are probably dealing with two separate entities when we are considering the specific retinal and renal lesion and the lesion in the lower extremity which by no means that I myself know of can you distinguish from senile arterial change

LEVINE I should like to draw your attention to a piece of work which was done by a Japanese pathologist The results have been bothering me ever since I read the paper and I would like especially those people like Dr Jackson and Professor Hoet, who are interested in hereditary and con genital aspects to throw some light on su h data

Dr Okomoto was interested in the relationship of alloxan to the zinc content of the islets, and he worked on a theory of zinc complex forma tion as a mode of alloxan action Having few rabbits to work with, he

used rabbits that had recovered from alloxan diabetes or those that were not successfully alloxanized He bred them in order to produce more rabbits — a very worthy enterprise To his astonishment he found that when the progeny grew up they required much less alloxan to become diabetic than did the fathers and mothers In the F 3 and F 4 generations there was a 30 per cent spontaneous hyperglycaemia and glycosuria Being a pathologist he measured the volume of the islets and there was a consistent decrease from one generation to the next Since he came from Japan and not from Lysenko territory I was puzzled about this

I have been urging people in the United States and elsewhere to let a geneticist have a go at it' Similar observations have been recorded by German observers in 1952 in studies on alloxanized rats I should like to hear Dr Jackson s and Professor Hoet s comments on these papers

SMART Work which was carried out in the Royal Air Force during the war did not indicate that vitamin-A deficiency was a limiting factor in raising the dark-adapted rod threshold Groups were observed who were receiving supplements of vitamin A but who nevertheless had raised dark-adapted rod thresholds Also substances such as alcohol which alter the pupil size can alter the value for the dark-adapted rod threshold as it is usually measured I have measured the dark-adapted rod threshold in many cases of steatorrhoea in which condition there is mal absorption of vitamin A and I have not found it to be significantly raised though this is possibly because sufficient B-carotene may be absorbed

Can Professor Hoet tell us the effect of normal pregnancy and of giving insulin to normal people on the dark-adapted rod threshold?

JACKSON In agreement with Professor Tunbridge and Dr Oakley certainly we must control our patients blood sugars at the present time but I tried to suggest that there may be other things more important to control which we do not yet know I am similarly in agreement that the exact categorization of all diabetics is impossible but nevertheless the general concept of the classification as I have outlined is very important for many reasons

As regards the validity of the diagnosis of pre-diabetes I would point to our own small series of women who gave rise to infants with enlarged pancreatic islets — seven out of twelve of them are already diabetic after 5 years while of those diagnosed in 1952 on the basis of obstetric sus picion and slightly abnormal glucose tolerance all five of those whom we have been able to follow up have become diabetic within 5 years There are several other published papers illustrating the progression of pre diabetes to overt diabetes including the recent work of Fajans and Conn and of Wilkerson I believe that temporary diabetes is merely a phase in the life history of a diabetic when some particular

to light the latent pre-diabetic abnormality Patients who s[1] state must be considered to be potential permanent diabetics up and treated accordingly

Professor Young asked for a definition of pre-diabetes While I really think of any real dividing line between pre-diabetes and d... yet for general discussion we keep them distinct I will try a definition 'The state in which evidence of carbohydrate abnormali not clinically apparent, but in which certain rather specific stresses produce certain specific abnormalities which we know to be related the later development of overt diabetes

Finally Dr Levine's extraordinary animals which appeared to c neo-Lamarckianism. It is conceivable that the alloxan had damaged th pancreas subclinically so to speak so that the extra stress of pregn rendered it partially inadequate The abnormal intra uterine environment then produced damage to the foetal pancreas (perhaps as in the human) so that one could thus imagine the appearance of diabetes in later generations This is merely a hypothetical alternative to a genetic one

YOUNG Thank you Dr Jackson. I think I was rather intrigued by the statement in your abstract that diabetes really exists from birth — that is pre-diabetes is it?

JACKSON In ordinary terminology yes

HOET Three points may be commented on as a result of this discussion

1 Professor Tunbridge brought forward in some way an opposition between some form of 'transit diabetes and permanent diabetes This is the problem of the beginning of diabetes mellitus and their relationship with the classical clinical picture of irreversible permanent overt diabetes mellitus accompanied by more or less severe complications From another viewpoint, it is the question when has diabetes mellitus in a transitory form to be controlled with insulin Is it possible to prevent the irreversible metabolic decompensation? We consider each metabolic disturbance caused by an infection, a mental stress trauma or burns as due to some transitory insufficiency of the islet function This is also the case in mild forms of pre-diabetes during pregnancy Although transitory diabetes may be recovered from without any anomaly of the G T T it appears to us that it has to be considered as a primary phase of diabetes mellitus although it may proceed for more than 20 years before overt irreversible diabetes mellitus results Therefore dietary and insulin treatment is justified in those pre-diabetes phases more so than they appear frequently in the offspring or sibs of more severe diabetics We suggest that immediate control of any mild disturbance of carbohydrate metabolism has to be put in action, so as to shorten the decompensated phase and to prevent further evolution to more severe and longer periods of diabetes mellitus. For each known case of diabetes mellitus there is a

patient with a concealed form waiting for some or other complication to be a recognized case Our treatment must protect the cases of potential *diabetes*

2 Dr Levine did mention the experiments of Japanese investigators on the offspring of alloxanized pregnant rats The unfavourable maternal environment has produced newborns *which later in life become* glycosuric even in the offspring of the second generation Bartelheimer and Kloos (*Zeitschr fur die Ges Experim Medizin* 1952 119 -46-65 Die Auswirkung des experimentellen Diabetes auf Graviditat und Nach kommenschaft) have seen hyperglycaemia and glycosuria in some cases with gigantism in second and third generations of mild alloxanized mothers Dr Haumont in experiments in collaboration with Goormagh *tigh and Hoet has treated pregnant rats with rather high doses of cortisone* from the eighth to the eighteenth day of gestation A percentage of resorptions are seen but in the living offspring urogenital malformations in the females were observed In the following generation this means rats born of mothers not treated but being the daughters of cortisone-treated rats Haumont has seen spontaneous cataracts and after 6 to 8 weeks hyperglycaemic phases occurred see preliminary paper in *Annales d endocrinologie* 1959 19 No 2 pp 442 5 'Effects de la Cortisone administree pendant la gestation

Therefore we stress the importance of a normal favourable maternal *environment in every case* but more so when some pathological genetical trend may be suspected

3 A colleague of Manchester did recall that night pilot candidates were investigated about their night vision On nutritional deficiency it has not been easy to discover clear cut cases of night blindness Environ mental factors or pupil dilatation or use of alcoholic beverages may play a role in ascertaining the dark-adaptation level We have been studying metabolic carotene *disturbances depending on insulin deficiency* or hypothyroidism There was no nutritional deficiency We demonstrated the correction in one and the same patient of the defective night vision by aetiological treatment with insulin or thyroid hormone The favourable response was seen in 3 days in selected fresh cases of pre-diabetes This insulin action is seen also in pre-diabetes of pregnancy It is an indication of a metabolic action of the hormone which is of paramount importance for the placenta and *foetal tissues and harmonious foetal* development

CONCLUDING REMARKS

F G YOUNG

YOUNG The last item on the programme is Ge- and conclusions by F G Young I am very hesitar general conclusions from the last two days but I thi number of points that have emerged which strike particularly interesting

One must necessarily be humble at the amount that know about insulin and diabetes but I recall Cla remark that what we believe we know is often a hin knowing the truth. I and perhaps others have found d understand a good deal of what I thought I knew before d

A point that has interested me very much in the di have had is the number of times the mechanism of action has been linked to high energy nitrogenous phosphate This is an idea which is clearly in the minds of several workers and it may well be that it will be a most fruitful years to come But as Dr Cori said we know little or nothin the mechanism of sugar transport and little or nothing abo mechanism of action of insulin We must still agree after d matters of this sort for two days that this is still true

On the other hand we have agreed about much We have the work of Dr Moloney on the production of insulin antib supported confirmed and used as in other laboratories while t is considerable agreement as to the importance of insulin in biosynthesis It is clear too that the existence of t e insulin which are being investigated is of fundamental sign in the growth of knowledge of the mechanism of action of insul and of diabetes and that these substances complicate the b of insulin The bio-assay of this hormone is a matter that needs m further investigation before it is on a satisfactory basis But me of different sorts are being brought to bear on this problem and knowledge grows particularly as knowledge of the action of insulin antagonists grows so I think we shall have more understanding of the cause of diabetes and the mechanism of action of insulin

I very much enjoyed the paper on salicylates and aspirin by Dr

Reid It is not unreasonable to say that one of the important re of the discovery by Hench of the remarkable effect of rheumatoid arthritis has been that for the first time a thoro investigation of the therapeutic effects of salicylates has been made Salicylates had been available for 80 years or more although thorough clinical trials of their actions and effectiveness had not been undertaken until recently and as Dr Reid said the more is known the more we are astonished at the effectiveness of this comparatively simple chemical molecule salicylic acid and its simple derivative aspirin

I would like to take the opportunity of expressing the appreciation of us all to the two gentlemen who provided papers as substitutes Dr Beigelman and Dr Levine I am sure that those of us who know Professor Loubatieres will very much regret his absence and its cause but his research has been most ably surveyed by Dr Levine

I would again like to thank our hosts for having made this meeting possible Dr Hobday mentioned in his opening address that I had been concerned in the proposal that this meeting should be held In this connection I rather feel like the man who said to his neighbour in a London Club I had no difficulty at all when we had twins! It has been no difficulty at all to me but I would like to emphasize how hard Dr F W Wolff in particular has worked on the arrangements for this meeting He has written innumerable tactful letters and he has handled all the arrangements remarkably effectively I have discussed matters with him once or twice but he and of course the Committee of the British Insulin Manufacturers behind him, have been responsible for all the details in a most effective manner

SUBJECT INDEX

AUTHOR INDEX

Page number in heavy type indicates one of the principal communications other page numbers refer to the discussions